

Studies in Marine Natural Products:  
Biologically Active Compounds from the  
New Zealand Algae and Invertebrata

A thesis  
submitted in partial fulfillment  
of the requirements for the Degree of  
Doctor of Philosophy in Chemistry  
in the University of Canterbury  
by

John D McCombs  
University of Canterbury

1989

copy 2 with 2 separate items  
in back pocket

## Contents

---

Abstract	1
Acknowledgements	2
Introduction	3
Discussion	
Bioactive Compounds - Initial Studies	24
Compounds from <i>Tedania</i> Species	34
Thyrsiferol	63
New Butenolide Dimers from <i>Delisea elegans</i>	96
Crystal Structure of Eudistomin K	100
Crystal Structure of Discorhabdin C	105
MassCalc - A Computer Program for Mass Spectrometry	109
Experimental	
General	115
Bioactive Compounds - Initial Studies	117
Compounds from <i>Tedania</i> Species	124
Thyrsiferol	142
New Butenolide Dimers from <i>Delisea elegans</i>	148
Crystal Structure of Eudistomin K	150
Appendix A - MassCalc User Manual	151
Appendix B - Atom Coordinates and Tables for Crystal Structure	172
Appendix C - Publications	185
Appendix D - Floppy disk 1 : MassCalc	
Appendix E - Floppy disk 2 : Other Programs	

## ***Tables and Illustrations***

---

Map of Collection Sites	15
Table of <i>in vitro</i> Activity versus Phylum	17
Graph for Reverse-phase Trial Column	28
Separation Tree for the PML1-6 Extract <sup>1</sup>	40
Separation Tree for the 1kg Extract of 5P2-24 <sup>1</sup>	42
Separation Tree for the Extract of 5P2-24 and 5P3-1 <sup>1</sup>	45
HPLC Chromatograms of T14.22 - T14.35	54
DCI/Ammonia Mass Spectra for T14.23, T14.33 and T14.34	57
<sup>1</sup> H and <sup>13</sup> C NMR Spectra of Thyrsiferyl 18-Acetate	69
COSY and DQCOSY Spectra of Thyrsiferyl 18-Acetate	70
HETCOR and XCORFE Spectra of Thyrsiferyl 18-Acetate	71
Selected nOe Difference Spectra of Thyrsiferyl 18-Acetate	72
Chemical Shift Assignments for Thyrsiferyl Acetate and Thyrsiferol	75
NMR Correlation Table for Thyrsiferyl 18-Acetate <sup>1</sup>	76
NMR Correlation Diagram for Thyrsiferyl 18-Acetate	77
Crystal Structure of Thyrsiferyl 18-Acetate <sup>1</sup>	78
Measurements from the Conformational Analysis of Thyrsiferyl Acetate	88

---

<sup>1</sup> Repeated in the fold-out section at the rear of this thesis

## Abstract

---

In the search for new classes of compounds with antiviral, antitumour or antibacterial activity, a number of benthic marine species were examined.

The extract of *Tedania connectens* was found to contain three compounds with intense *in vitro* antiviral and cytotoxic activity ( $IC_{50}$  against P388 murine leukemia cells was 20-40 pg/ml). Examination of the mass spectra of these compounds indicated the presence the known compound tedanolide, together with a deoxy and a chlorinated derivative.

Attempts to convert the antiviral and cytotoxic compound thyriferol to thyrifer-18-one and to the 18-epimer were unsuccessful. However, the  $^1H$  and  $^{13}C$  NMR spectra of thyriferyl acetate, including all the *pro-r* and *pro-s* proton resonances, were completely assigned using a combination of 2D NMR spectroscopy and molecular mechanics calculations.

Two new dimeric butenolides were isolated from the red alga *Delisea elegans*, and their structures determined by single X-ray crystallography. The crystal structure of discorhabdin C, a cytotoxic pigment from a sponge of the genus *Latrunculia*, was determined, as was the crystal structure of the *p*-bromobenzoyl derivative of the known compound eudistomin K.

Several computer programs were written to assist data analysis. The program "MassCalc" was written to free the chemist from the tedious computational tasks usually associated with interpreting mass spectra. A group of five programs were written to simplify the interpretation of the results of molecular mechanics and X-ray crystallography calculations.



## ***Acknowledgements***

---

I am indebted to my supervisors, Drs Murray Munro and John Blunt, not only for their advice and encouragement throughout this project, but for their efforts in creating the environment which made this work possible.

I wish to thank Dr Ward Robinson for assistance and advice with the X-ray crystallography, Dr Jim Coxon who made available the molecular mechanics software used for work described in this thesis, Vicky Calder and Gill Barns for the bio-assays and Drs Graham Fenwick and Chris Battershill for their assistance in collecting and identifying the organisms studied. I am grateful to the technical staff of the Chemistry Department for their helpfulness and expertise.

I would also like to thank Dr Lewis Pannell, of NIDDK, National Institutes of Health, for mass spectra, and Varian (Australia) for the 500 MHz NMR spectra.

## Introduction

---

The sea has long been a source of fascination. For the natural products chemist it represents a rich source of interesting compounds and the last twenty years has seen a great increase in the number of new structures reported. Prior to this, the relative inaccessibility of benthic marine flora and fauna has limited the number of species and amount of material available for natural products isolation work. It was the development of SCUBA diving that permitted ready and reliable access to the species down to 40m. An appreciation of the impact of this technique, in facilitating chemical studies, may be gained from even the most cursory examination of the marine natural products literature.

Although many studies have been undertaken with the simple aim of isolating new chemical structures or understanding the taxonomic and ecological relationships between species, the search for potential pharmaceuticals is, increasingly, the driving force for this type of research.

The use of natural products for medicinal purposes is well documented in most civilisations and periods of history. Today many common drugs are either natural products or have their origins in terrestrial plants or micro-organisms. Aspirin, penicillin, morphine and digitalis are just a few of many examples.

The oceans cover nearly three-quarters of the earth's surface, and it has been estimated that more than eighty percent of the earth's 500,000 species live in the marine environment. In addition many groups of organisms, such as the sponges (Porifera) and ascidians (Ascidiacea), are solely aquatic.<sup>1</sup> Given the huge diversity of life in the seas, and the precedent of drugs which have been derived from

---

1 der Marderosian, A.D., *J. Pharm. Sci.*, **58**, 1 (1969).

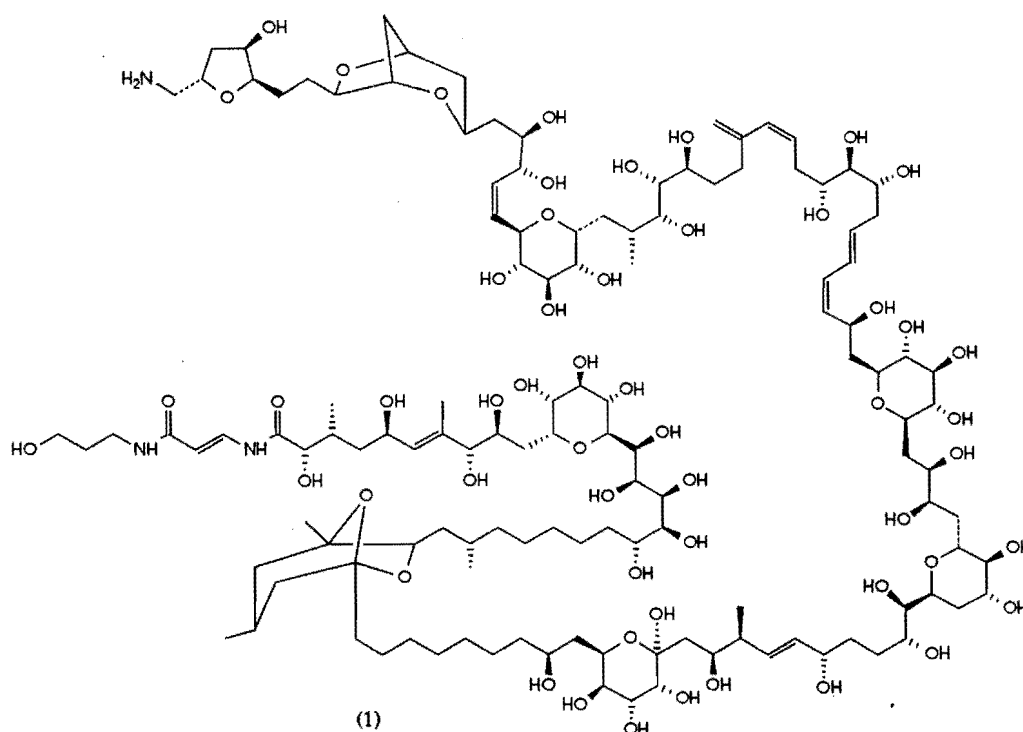
terrestrial sources, a case for screening marine organisms can be made on purely statistical grounds.

There have been many excellent reviews of the compounds isolated from marine species. Amongst the most thorough are those of Faulkner<sup>2</sup> in which the natural products are presented by the taxonomic group from which they were obtained. Other reviews such as those in the monographs edited by Scheuer<sup>3</sup> have been oriented to particular types of biological activity, groups of organisms, or topics, such as ecology or chemotaxonomy. The marine natural products with cytotoxic, antitumour and antiviral activity were reviewed by Munro *et al.*<sup>4</sup> The following section describes several compounds which were isolated from four marine species. These novel substances serve to illustrate the potential of marine organisms as a source of new classes of biologically active agents.

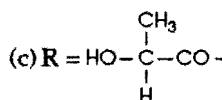
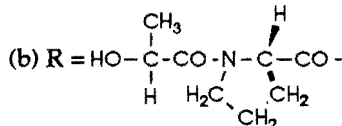
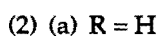
Palytoxin (1) is a remarkable compound, both for its high level of cytotoxicity and for the complexity of the structure. It was first isolated from the zooanthid *Palythoa toxica* in 1971<sup>5</sup> and the complete structure elucidation was reported ten years later,

- 
- 2 Faulkner, D.J., *Tetrahedron*, **33**, 1421 (1977), Faulkner, D.J., *Natural Product Reports*, **1**, 251 and 551 (1984), Faulkner, D.J., *Natural Product Reports*, **3**, 1 (1986), Faulkner, D.J., *Natural Product Reports*, **4**, 539 (1987), Faulkner, D.J., *Natural Product Reports*, **4**, 613 (1988).
  - 3 Scheuer, P.J., *Marine Natural Products: Chemical and Biological Perspectives*, Vols I - V, (Academic Press).
  - 4 Munro, M.H.G., Luijbrand, R.T. and Blunt, J.W., *The Search for Antiviral and Anticancer Compounds from Marine Organisms in Bio-organic Marine Chemistry* (Springer Verlag : New York 1987).
  - 5 Moore, R.E. and Scheuer, P.J., *Science*, **172**, 495 (1971).

by two independent groups. The structure of palytoxin and the stereochemistry of the sixty-four chiral centres were determined by spectroscopic and chemical methods.<sup>6,7,8</sup> Although palytoxin is the most toxic marine natural product known ( $LD_{50}$  0.15  $\mu$ g/kg in mice), its non-selective cytotoxicity means it has limited potential as an antitumour agent.<sup>9</sup>



- 
- 6 Moore, R.E. and Bartolini, G., *J. Am. Chem. Soc.*, **103**, 2491 (1981).
  - 7 Uemura, D., Ueda, K., Hirata, Y., Naoki, H. and Iwashita, T., *Tetrahedron Lett.*, **22**, 2781 (1981).
  - 8 Moore, R.E., Bartolini, G., Barchi, J., Bother-By, A.A., Dadok, J. and Ford, J., *J. Am. Chem. Soc.*, **104**, 3776 (1982).
  - 9 Hashimoto, Y., *Marine Toxins and other Bioactive Marine Metabolites*, 248 (Japan Scientific Societies Press : Tokyo 1979).

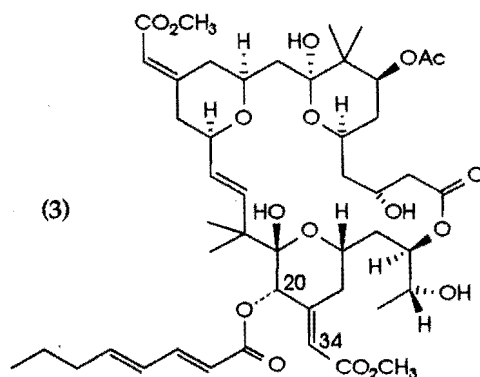


10 Rinehart, K.L., Gloer, J.B., Cook, J.C., Mizesak, S.A. and Scahill, T.A., *J. Am. Chem. Soc.*, **103**, 1857 (1981).

11 Montgomery, D.W. and Zukoski, C.F., *Fed. Proc.*, **42**, 374, (1983).

melanoma cell lines. Didemnin B is especially noteworthy as it is the first marine compound to enter human clinical trials.<sup>12</sup>

Extracts of the bryozoa *Bugula neritina* and *Amathia convoluta* were found to have significant *in vivo* antitumour activity. Over a period of sixteen years bryostatin 1 (3) and eighteen other related compounds, bryostatins 1-17 and bryostatins A and B, have been reported.<sup>13,14</sup> Two of the bryostatins were obtained from the sponge *Lissodendoryx isodictyalis* which was later found to contain *B. neritina* in the sponge matrix.<sup>15</sup> The structure of bryostatin 1 was determined by single crystal X-ray crystallography after the pure compound had been isolated from 500kg of the animal, using silica gel and gel permeation chromatography. The typical concentration of the bryostatins in the wet animal is about one part in 10.<sup>8</sup>

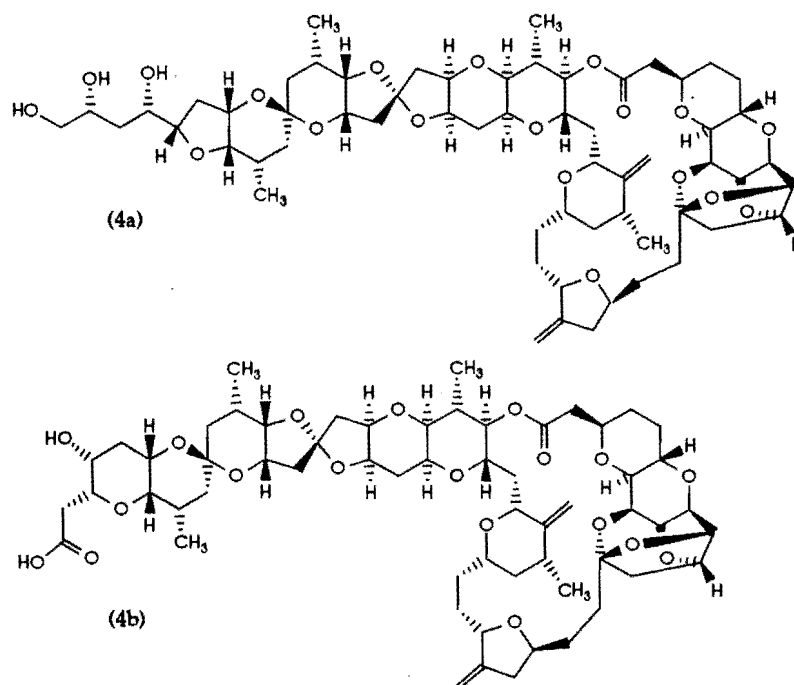


- 12 Suffness, M. and Thompson, J.E., *National Cancer Institutes Role in the Discovery of New Antineoplastic Agents in Memoirs of the California Academy of Sciences*, **13**, 151 (California Academy of Sciences : San Francisco 1988).
- 13 Pettit, G.R., Herald, C.L., Doubek, D.L., Herald, D.L., Arnold, E. and Clardy, J., *J. Am. Chem. Soc.*, **104**, 6846 (1982).
- 14 Pettit, G.R., *et al* in: *J. Nat. Prod.*, **46**, 917 (1983); *J. Nat. Prod.*, **46**, 528 (1983); *J. Org. Chem.*, **48**, 5354 (1983); *J. Am. Chem. Soc.*, **106**, 6768 (1984); *J. Nat. Prod.*, **47**, 941 (1984); *Can. J. Chem.*, **63**, 1204 (1985); *Tetrahedron*, **41**, 985 (1985); *J. Nat. Prod.*, **49**, 661 (1986); *J. Org. Chem.*, **52**, 2848 (1987); *J. Org. Chem.*, **52**, 2854 (1987).
- 15 Pettit, G.R., Kamano, Y., Herald, C.L., Schmidt, J.M. and Zubrod, C.G., *Pure and Appl. Chem.*, **58**, 415 (1986).

The origin of the bryostatins has been the subject of debate. The geographic distribution of the compounds has been cited as evidence for a dietary source, but it may reflect genetic or environmental differences. Steroids, characteristic of dinoflagellates, have been isolated from *B. neritina* which, it is suggested, indicates a symbiotic relationship between the bryozoan and a dinoflagellate.<sup>16</sup> Ecological and chemical studies are required to confirm these assertions.

The bryostatins are presently candidates for clinical testing by the American National Cancer Institute; however sufficient material has yet to be obtained.<sup>17</sup>

The observation that extracts of the Japanese sponge *Halichondria okadai* exhibited strong *in vivo* antitumour activity led to the isolation of the halichondrins.<sup>18</sup> Of the eight compounds reported, halichondrin B (4a) has the greatest *in vitro* activity against B16 melanoma cells ( $IC_{50}$  93pg/ml). Life extensions of up to 200% and



16 Pettit, G.R., Kamano, Y. and Herald, C.L., *J. Nat. Prod.*, **49**, 661 (1986).

17 Suffness, M. and Thompson, J.E., *National Cancer Institutes Role in the Discovery of New Antineoplastic Agents in Memoirs of the California Academy of Sciences*, **13**, 151 (California Academy of Sciences: San Francisco 1988).

18 Hirata, Y. and Uemura, D., *Pure and Appl. Chem.*, **58**, 701 (1986).

300% were reported for *in vivo* tests in B-16 melanoma and P388 murine leukemia systems, respectively.

The compounds were isolated using liquid/liquid extraction, gel permeation and reverse-phase chromatography, and the structure, including the absolute configuration, of the *p*-bromophenacyl ester of norhalichondrin A (4b) was determined by single crystal X-ray crystallography. The structures of the remaining compounds were determined by spectroscopic means.

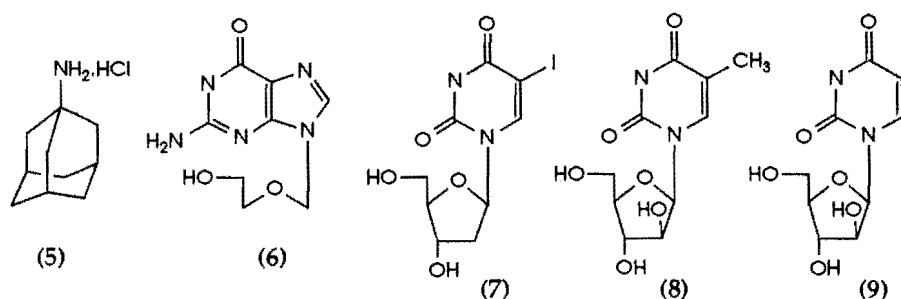
One of the major goals of the marine chemistry group at the University of Canterbury has been the isolation and structure determination of new classes of compounds with antiviral and antitumour activity. The work described in this thesis was a part of that project.

The development of antiviral compounds has been a difficult problem. Viruses reproduce by subverting the host cell's mechanisms for protein synthesis and DNA and RNA replication, hence an antiviral chemical must either prevent the virus attacking the host cell, must be selectively toxic to infected cells, or must prevent new viruses leaving the cell in a viable state. The similarity between a normal host cell and an infected cell is the reason that relatively few antiviral drugs have been developed.

For similar reasons there is no anticancer drug which shows selectivity towards tumour cells that is comparable to the selectivity obtained by modern antibiotics. The successes in cancer chemotherapy have been largely confined to those types with rapidly dividing cells. Compounds which inhibit cell growth or cell division, for example the didemnins, may show both antiviral and antitumour activity. In this thesis, the term antitumour is used to refer to *in vivo* activity, while cytotoxicity refers to *in vitro* activity.



With the exception of amantadine (5), all of the currently available antiviral drugs are synthetic nucleosides. Of these only acyclovir (6) is free from significant side-effects, and some, such as idoxuridine (7), are too toxic for anything except topical use.<sup>19</sup> It is interesting to note that origins of the nucleoside antiviral drugs may be traced back to the natural products, spongothymidine (8) and spongouridine (9), which were isolated from a Caribbean sponge.<sup>20</sup>



#### Sources of and Reasons for the Activity

The source of many of the marine natural products which have been reported is unclear. The compounds may be metabolites of the organism under examination, they may be metabolites which have been concentrated from a dietary source, or they may result from contamination of the sample by other species which live in close association with organism. Undoubtedly some of the reported compounds were artefacts of the isolation procedures.

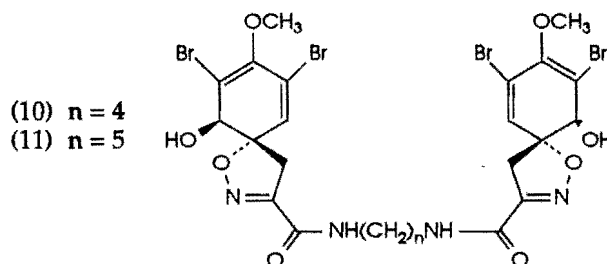
There are, however a number of studies in which the true source of the compounds was established. Examples from each of the listed categories can be found. For example, cell type fractionation and X-ray micro analysis was used to show that

---

19 Bean, B., *Clinical Microbiology Newsletter*, 4, 64 (1982).

20 Bergman, W. and Feeney, R.J., *J. Org. Chem.*, 16, 981 (1951).

the compounds aerothionin (10) and homoaerothionin (11) were located within a single cell type of the sponge from which they were isolated.<sup>21</sup> In contrast a series of diketopiperazines, which were reported from the sponge *Tedania ignis*,<sup>22</sup> were



shown to be metabolites of a bacteria species, *Micrococcus* sp., which was consistently associated with the sponge.<sup>23</sup> Similarly, there is evidence that palytoxin (1) is a metabolite of a symbiotic bacterium.<sup>24</sup> Perhaps the best known example of an organism which concentrates compounds from a dietary source is the sea hare *Aplysia californica* (Mollusca). This animal was found to accumulate a range of halogenated monoterpenes from the red alga, *Plocamium cartilagineum*, on which it grazed. Over twenty nudibranch species are known to contain dietary metabolites from a variety of sources, including sponges and bryozoa.<sup>25,26</sup>

The exact function of these secondary metabolites is also far from clear. It has frequently been suggested that the compounds which exhibit cytotoxic, antiviral

21 Thompson, J.E., Barrow, K.D. and Faulkner, D.J., *Acta Zool. (Stockh.)*, **64**, 199 (1983).

22 Schmitz, F.J., Vanderah, D.J., Hollenbeak, K.H., Enwall, C.E.L., Gopichand, Y., Sengupta, P.K., Hossain, M.B. and van der Helm, C.J., *J. Org. Chem.*, **48**, 3941 (1983).

23 Stierle, A.C., Cardellina, J.H. and Singleton, F.L., *Experientia*, **44**, 1021 (1988).

24 Moore, R.E., *Pure and App. Chem.*, **54**, 1919 (1982).

25 Faulkner, D.J. and Stallard, M.O., *Tetrahedron Lett.*, 1171 (1973).

26 Stallard, M.O. and Faulkner, D.J., *Comp. Biochem. Physiol.*, **49B**, 25 (1974).

and antimicrobial activity have a protective role. Benthic organisms must defend themselves from encroachment by surrounding species, and there is the constant threat of infection by micro-organisms. This proposition is supported by a preliminary examination of the marine chemistry group's collection which indicated a much higher incidence of *in vitro* antiviral and antitumour activity in encrusting and encroached massive sponges, than in species that were not challenged for space.<sup>27</sup> Similarly members of the red algae family Bonnemaisoniaceae, observed at Kaikoura, are generally free from epibionts. Extracts of species in this family are known to contain compounds with strong antimicrobial activity.<sup>28</sup>

### Collections & Methods

The results of work by two other groups have influenced the development of the collection strategy used at the University of Canterbury. Rinehart's group, from the University of Illinois, had expeditions in the *RV Alpha Helix* to Baja, California (AHBE) and the Caribbean (AHCE), in 1974 and 1978 respectively.<sup>29</sup> A notable feature of these expeditions was the inclusion of shipboard assays for biological activity, which enabled the immediate re-collection of species that were found to be active.

In the first collection some 831 species were collected from the coast of Baja, California, and assessed for *in vitro* antimicrobial activity with the shipboard assays. A limited number of the extracts were later tested for antiviral and

- 
- 27 Battershill, C., *The search for Antiviral Chemicals from New Zealand's Marine Benthos - Ecological Clues*, Paper presented at the NZ Marine Sciences Society Conference (1986), and personal communications.
- 28 McConnell, O.J. and Fenical, W., *Antimicrobial Agents from the Marine Red Algae Family Bonnemaisoniaceae in Marine Algae Pharmaceutical Science* (Ed Hoppe, H.A. and Tanaka, Y.) 403 (de Gruyter: Berlin 1979).
- 29 Rinehart, K.L., Shaw, P.D., Shield, L.S., Gloer, J.B., Harbour, G.C., Koker, M.E.S., Samain, D., Schwartz, R.E., Tymiak, A.A., Weller, D.L., Carter, G.T., Munro, M.H.G., Hughes, R.G., Renis, H.E., Swynenberg, E.B., Stringfellow, D.A., Vavra, J.J., Coats, J.H., Zurenko, G.E., Kuentzel, S.L., Li, L.H., Bakus, G.J., Brusca, R.C., Craft, L.L., Young, D.N. and Connor, J.L., *Pure & Appl. Chem.* **53**, 795 (1981).

antitumour activity. The Caribbean expedition, which visited sites from Panama to Mexico, yielded over 600 species. Shipboard assays for antimicrobial, antiviral and antitumour activity were carried out.

Although the portion of the species from the AHBE collection which were submitted for antiviral and antitumour testing was small, the number assayed against the KB nasopharyngeal cell line was sufficient to indicate some trends. It was found that nearly a third of the extracts of the Porifera, Cnideria and Echinodermata exhibited some activity, whilst the remainder showed an incidence of activity of less than ten percent. The assay results from the AHCE collection showed a high incidence of antiviral and cytotoxic activity in extracts of the Porifera, Cnideria, Chordata and Phaeophyta. Several phyla, such as the Cyanophyta had a high incidence of activity, but the sample size was very small.

The strongly biologically active eudistomins and the didemnins were amongst the compounds which were isolated from species collected during these expeditions.

The Roche Institute of Marine Pharmacology, based near Sydney, Australia, was established for "*the discovery and structural elucidation of marine natural products with biological activity.*"<sup>30</sup> This group mainly restricted its investigations to species of the Porifera, Algae and Coelenterata which were available in quantity, and in many cases compounds with no reported biological activity were isolated. It appears that compounds were often assayed only after the purification was complete.<sup>31</sup> In the light of recent studies which have attempted to relate the presence of biological activity to factors such as environmental crowding, this approach may have excluded many active species, which were available only in small quantities.

Since 1982 members of the marine chemistry group at the University of Canterbury have made collections from the sub-tropical waters of northern New Zealand, to

---

30 Wells, R.J., *Pure and Appl. Chem.*, **51**, 1829 (1979).

31 Jacobs, R.S., Culver, P., Langdon, R., O'Brien, T. and White, S., *Tetrahedron*, **41**, 981 (1985).

the sub-antarctic conditions of the Auckland Islands. Over 3500 specimens have been collected, representing 1000 species. The collection sites for the material examined in this thesis are shown overleaf

The first step of the isolation cycle is the ongoing process of collecting as wide a range of organisms as possible. Methanol/toluene extracts of these samples are then submitted for screening in the *in vitro* antiviral and cytotoxicity assays. Samples which are selected for further work, on the basis of the screening results, are recollected in bulk, extracted and re-assayed. If suitable biological activity is obtained in this second step, and an adequate supply of the organism is available, isolation of the active components may be undertaken. *In vivo* assay of the partially purified extract is desirable.

The collection methods fall into three groups. The easiest area to collect specimens is the intertidal region. The majority of species collected from this habitat are algae, but a few sponge and ascidian species are also present.

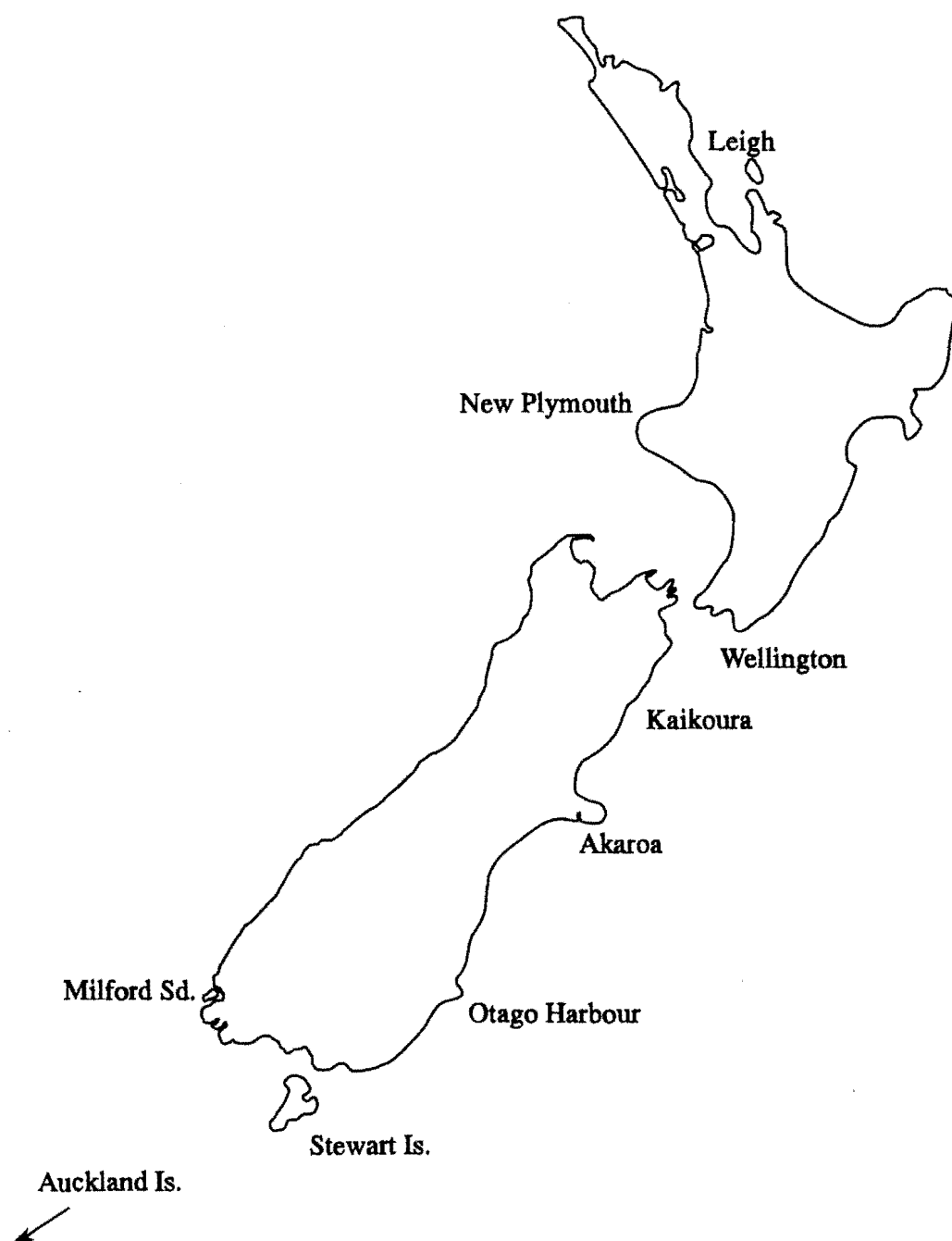
Deep water species were obtained by dredging from the Government research ships the *James Cook*, near the Otago Peninsula, and the *Tangaroa*, off the Kaikoura coast. These vessels enabled the collection of material from a variety of bottom types, at depths from 30m to 2500m.

By far the majority of specimens have been collected by SCUBA diving from inshore waters, at depths of up to 40m. Of the three collection techniques, SCUBA diving is the most satisfactory. Unlike dredging, hand collection offers the opportunity to sample an area much more thoroughly, and the chances of recollection are much enhanced. The samples may be collected intact, and the individuals may be bagged separately to prevent cross-contamination of samples.

Once collected, the samples were sorted by species, and two sub-samples of each specimen were taken. One sub-sample, two grams, was taken for initial screening for biological activity, and the other was preserved as a voucher for future identification. Each sample was photographed with a colour chart and scale. Algae, cnidarians and ascidians were preserved in formalin/sea water, while the

## Map of Collection Sites

---



other phyla were preserved with propan-2-ol. The remainder of the specimen, and the assay sample were stored by freezing. The lower limit of sample size was about four grams, which was enough for an assay sample and a voucher specimen.

The main problem that arises at the collection time is that of ensuring sample homogeneity. Species may be difficult to distinguish. A good example of this problem is the *Tedania* genus, where the surface morphology varies greatly depending on environment. With the exception of *T. diversirhaphidiophora*, these species cannot be distinguished without microscopic examination of the spicule skeleton. The homogeneity of a sample may be compromised by contamination of specimens with the fluid that has leached from other species in the diver's collection bag. Placing individual specimens in plastic bags as they are collected is the only way to avoid this problem. Finally, there is the problem of species growing in close association with one another. This may occur from the relatively gross level of the polychaete worms which are very commonly found in sponges collected in harbours, to the tiny sponges which infest the bryozoa *Margaretta barbata*, to the bacteria and algae which frequently inhabit a sponge matrix. Ensuring that a sample is free of such organisms may be almost impossible. Where the contaminating organisms can be separated they have been assayed separately. Where such organisms exhibit activity, the question of which species is the source of the activity remains unclear.

The following table summarises the incidence of biologically active *species*, in the collection. Antimicrobial and P388 testing has lagged somewhat behind the antiviral testing, hence the lower numbers in the table. The large number of Porifera and Ascidiacea relative to the other phyla is the result of early targeting of these two groups, based on the results of Rinehart's ACBE and AHCE collections. More recently collections have included species from the other phyla. The NCI has

Table of incidence of *in vitro* activity versus phylum

Phylum	AV/Cyt Cyt <sup>a</sup>			P388 <sup>b</sup>		AM <sup>c</sup>	
	%	%	n	%	n	%	n
Porifera	37	47	563	52	275	28	302
Acideacea	28	35	165	64	79	41	80
Bryozoa	27	45	45	39	28	19	27
Cnideria	3	6	37	21	19	6	18
Mollusca	18	24	17	42	12	8	12
Echinodermata	63	74	19	33	12	38	13
Annelida	40	40	5	0	2	0	2
Brachiopoda	0	0	1	100	1	0	1
Chlorophyta	25	33	12	17	12	17	12
Phaeophyta	52	55	31	64	31	37	30
Rhodophyta	22	42	45	45	29	27	22

<sup>a</sup> Antiviral and/or cytotoxic response in the HSV 1/BSC cell system.

<sup>b</sup> Cytotoxicity against the P388 cell line.

<sup>c</sup> Response against any of *E. Coli*, *P. aeruginosa*, *B. subtilis* or *C. albicans*.

reported the incidence of *in vivo* P388 activity for these phyla to be from two to five percent<sup>32</sup>

### Taxonomy

Referring to sponges, Bergquist made the following observation:

*The classification of any group has a central rôle in a study in its own right, but also provides an indispensable framework into which workers from many disciplines may integrate their observations and thus attempt comparative studies. Unstable classification introduces uncertainties into all aspects of the group concerned.*<sup>33</sup>

This statement is certainly applicable to marine natural products research and may also be applied to other groups of marine organisms where there is difficulty in obtaining a reliable identification of specimens.

32 Suffness, M. and Thompson, J.E., *National Cancer Institutes Role in the Discovery of New Antineoplastic Agents in Memoirs of the California Academy of Sciences*, 13, 151 (California Academy of Sciences : San Francisco 1988).

33 Bergquist, P.R., *Sponges*, p136 (Hutchinson : London 1978).



Identification of samples is important for a number of reasons. Before embarking on a bio-assay directed isolation, identification of at least the family, and preferably the genus, of an organism is necessary to ensure effort is not devoted to re-isolating known compounds. Unfortunately there are many instances where compounds have been reported from wrongly identified material. For example the group of cytotoxic compounds known as the trinculins were reported from a sponge, which was incorrectly reported as *Latrunculia brevis*.<sup>34,35</sup> Where compounds have been reported from a species of interest, efforts should be made to obtain voucher specimens to allow comparison with the locally collected material.

Secondly, experience has shown that re-collection of a species on the basis of photographs may be unreliable due to the similarity in appearance of unrelated organisms. This is a particular problem with some groups of sponges and ascidians.

In the course of many expeditions it is inevitable that there will be duplication of species. It is essential to identify the replicates, both to avoid repetition of isolation work, and because examination of the screening results may give valuable information about the geographical and seasonal variation of the activity.

### **Screening and Biological Assays**

The biological assay methods are, of course, crucial in the process of isolating new compounds. There is a constant tension between the need to obtain as broad an assessment of an extract as possible, and the need to be able to cheaply and rapidly assess a large number of samples.

Typically extracts are assessed for the mode of biological activity that a particular research group is interested in. Extracts are screened initially with a few well chosen assays which are believed to be representative of the type of disease or pathogen of interest. For example Rinehart's AHCE collection used two viruses,

---

34 Capon, R.J. MacLeod, J.K. and Willis, A.C., *J. Org. Chem.*, **52**, 339 (1987).

35 Identified by Dr C.N. Battershill, personal communication.

*Herpes simplex* virus type I (HSV I) and vesicular stomatitis virus (VSV), to assess the antiviral activity. These were chosen as being representative of the two types of virus, DNA viruses and RNA viruses.

An *in vitro* screen using only a few organisms is inexpensive and rapid, but has two undesirable consequences. Firstly the models chosen will never be completely representative of the class of organisms, or tumours, they represent, and hence may give negative results for compounds that are worthy of attention. The use of L1210 and P388 murine leukemia cell lines illustrates this point. These two models which represent rapidly dividing cell types, have been used in many laboratories as a primary screen for anti-tumour compounds. Given that human tumours show marked differences in drug sensitivity, it is perhaps not surprising that these screens have led to the discovery of drugs which are effective against rapidly growing tumours, but which have little activity against the slow growing types.

Secondly an *in vitro* screen may not give a true indication of the activity a substance will exhibit in a whole animal; that is the assay system is likely to generate false positives. This problem is not as serious as the first, but it is an important factor in the light of the amount of effort that is required to isolate and determine the structure of a compound.

The American National Cancer Institute (NCI)<sup>36</sup> has developed a screening protocol to counter these problems. Their system uses an initial *in vitro* screen with up to a hundred tumour types and sub-types. The active extracts are then assessed with the same tumour types in the much more expensive and time-consuming *in vivo* system using xenografts in athymic mice.<sup>37</sup> Over a five year period the NCI hopes to collect nearly 38,000 species, including 5,000 from marine sources, for testing.

---

36 Suffness, M. and Thompson, J.E., *National Cancer Institutes Role in the Discovery of New Antineoplastic Agents in Memoirs of the California Academy of Sciences*, 13, 151 (California Academy of Sciences : San Francisco 1988).

37 Athymic mice ("nude mice") have a defective immune system which allows grafts of foreign tissue to be made without rejection.

Regrettably, few laboratories have the resources of the NCI. Since starting the search for biologically active compounds the marine chemistry group at the University of Canterbury has used four micro-organisms, *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Candida albicans*, and two virus, HSV I and Polio virus type 1 (PV1), *in vitro* assay systems for the initial screening. More recently the initial extracts have been tested in a P388 murine leukemia *in vitro* assay. Extracts with cytotoxic activity are also assessed in an *in vivo* system before bio-assay directed isolation work is attempted.

The antimicrobial activity of compounds is measured by placing an extract-impregnated paper disc (6mm diameter) on the surface of an agar plate which has been inoculated with the micro-organism in question. The plate is then incubated at 37°C for twenty-four to thirty-six hours. Any antimicrobial activity will cause a clear, micro-organism free, ring around the disc, the so called "zone of inhibition." The diameter of the zone of inhibition, when compared with that of the standard, gives an approximate indication of the level of the activity. Gentamycin, at 10µg per disk, is used as the standard for *E. coli* and *P. aeruginosa*, while chloramphenicol (30mg/disk) and nystatin (100 units) are used for the *B. subtilis* and *C. albicans* assays respectively. For some compounds, the size of the zone of inhibition is undoubtedly limited by diffusion. In practice, however, it was assumed that all compounds diffuse through the agar at equal rates.

The antiviral assay used monkey kidney cells (BSC, Vero or CV-1), grown on the surface of plastic wells, as hosts for the test virus. HSV 1 and PV1 were used to represent DNA and RNA viruses, respectively. The cells were grown in nutrient media until they became a monolayer. The cell sheet was then infected with the test virus, prior to introducing a sample-impregnated filter paper disc. After incubation, for a minimum of eighteen hours, the assays were read by microscopic examination. A six point scale was used to score the result. No activity, that is cytopathic effect (CPE), was observed over the whole cell sheet was marked as "-", while the complete absence of CPE was rated as "WW" or whole well activity. Intermediate levels of CPE were rated from "±", "+", "++" to "+++". The cytotoxic effect of the sample on the cell sheet was also scored by the same system. The

cytotoxic reactions were divided into seven sub-types (C1-C7) depending of the morphology of the affected cells. The cytotoxicity sub-type was generally consistent for a particular compound.

Unless otherwise stated, the antiviral activity is reported in this thesis in the form (X,Y,Z/C W), where "X" is the HSV-1 score, "Y" the PV1 and "Z" is the cytotoxicity. "C" is the optional cytotoxicity sub-type and "W" is the weight of sample that was impregnated on the disc, usually in micrograms. The symbol "?" is used to indicate that the activity or weight could not be determined, and "nd" denotes the activity for that virus type was not determined. Experience with this assay system has shown that the results should be regarded more as qualitative rather than quantitative. Some antitumour drugs which are protein synthesis inhibitors display antiviral activity in this assay.<sup>38</sup>

The P388 *in vitro* system is used as an indication of possible antitumour activity. The P388 cells are grown in suspension, and the sample is added as a methanol solution. A series of dilutions of each sample is made to allow the concentration at which the sample growth rate is half the control rate to be determined. This concentration is known as the IC<sub>50</sub> or ED<sub>50</sub>. The P388 test is very sensitive and responds to over ninety-five percent of the clinically effective antitumour drugs. An IC<sub>50</sub> of less than 30µg/ml for crude extracts is considered active. The P388 cell line may also be used as an *in vivo* assay system. In this case the results are expressed in terms of life extension compared with the control (T/C). A T/C of greater than 175% is considered significant activity, while a T/C of greater than 150% is considered as biologically significant.<sup>39</sup>

---

38 Copp,B.R., personal communication.

39 Suffness,M. and Douros,J., *J. Nat. Prod.*, 41, 1 (1982).

The crown-gall potato disc assay was evaluated as an alternative to the P388 system. This assay measures the ability of a sample to inhibit neoplastic disease induced, in potato discs, by inoculation with the bacterium *Agrobacterium tumefaciens*. A good correlation has been reported between the results of this assay system and those of the P388 *in vivo* system.<sup>40,41</sup> Unfortunately the assay could not be made to perform reliably.

### Work Done in this Thesis

The work reported in this thesis spans a number of areas. The main effort was devoted towards the isolation and structure determination of antiviral compounds. Several species were examined before a suitable candidate for the isolation work was found. Early exploratory studies on a sponge of the *Ircinia* genus was promising, but work was discontinued due to the difficulty in obtaining more sponge. A bryozoan, *Margaretta barbata* and the colonial ascidian *Aplidium gilvum* were then considered, but the antiviral and cytotoxic activity was found to be too unpredictable. An unidentified sponge from the Otago harbour, which was subsequently found to be a *Tedania* species, was finally selected on the basis of its antiviral activity and ready availability. Three strongly antiviral compounds were isolated in this bio-assay directed study.

In the months between making the first collections and obtaining the initial antiviral assay results two new dimeric butenolides were isolated from the red alga *Delisea elegans*. The structures of these unusual compounds were determined by single crystal X-ray diffraction studies.

The observation that the cytotoxic activity of the algal metabolite thyrseferol was greatly enhanced by oxidation<sup>42</sup> prompted a number of attempts at chemical

---

40 Galsky, A.G., Kozimor, R., Piotrowski, D. and Powell, R.G., *JNCI*, **67**, 689 (1981).

41 Ferrigni, N.R., Putnam, J.E., Anderson, B., Jacobsen, L.B., Nichols, D.E., Moore, D.S., McLaughlin, J.L., Powell, R.G. and Smith, C.R., *J. Nat. Prod.*, **45**, 679 (1982).

42 Thomas, F.N., BSc Hons project (University of Canterbury : 1986).

modification of this compound. As part of this study the complete  $^1\text{H}$ -NMR and  $^{13}\text{C}$ -NMR spectra were assigned, and molecular mechanics calculations were performed in an attempt to understand the three-dimensional conformation.

The crystal structures of two natural products, isolated by other members of the marine chemistry group, were determined. The structure of a highly cytotoxic, red-brown pigment, named discorhabdin C, from an undescribed *Latrunculia* species could not be elucidated by spectroscopic means. The structure of the cytotoxic and antiviral compound, eudistomin K, was determined as the *p*-bromobenzoate.

Finally several computer programs were written to facilitate data analysis. The program MassCalc is an intelligent "calculator," designed to aid the chemist interpret mass spectra. A group of programs, called XCalc, XGeom, XLat, Boltz and XConf, were written to assist the analysis of the results of the molecular mechanics and single crystal X-ray diffraction studies.

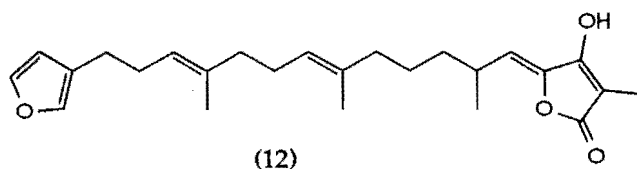
### Bioactive Compounds - Initial Studies

---

The first collection expedition, involving dredging and SCUBA diving near the Kaikoura peninsula, yielded 449 samples, the majority of which were sponges or ascidians. *In vitro* assays of the extracts of these samples, against HSV-1 and VSV,<sup>43</sup> revealed sixteen extracts with antiviral activity and no cytotoxicity, and eleven extracts with antiviral and cytotoxic activity. A further nineteen extracts had only cytotoxic properties. Three species were selected for initial investigations, on the basis of their biological activity and sample availability.

The first species considered was a sponge, subsequently shown to be *Ircinia* sp. A (order Dictyoceratida, family Thorectidae, voucher number U238-6), collected by dredging (300m), from the *RV Tangaroa*, at a point south of the Kaikoura peninsula. Although it was difficult to obtain, and had to be dissected away from the mass of worm tubes in which it grew, it displayed strong antiviral activity with no cytotoxicity.

Initial fractionation using multi-solvent partitioning techniques gave variable results in terms of the biological activity. The antiviral activity decreased, while the fractions that were active became cytotoxic. For this reason work on the species was abandoned. This was fortunate as the known compound variabilin (12) was



subsequently shown to be responsible for the antiviral activity in the sponge *Ircinia*

---

43 Assays were provided by Dr R.G. Hughes, Roswell Park Memorial Institute, Buffalo, New York.

sp. B, collected from the Otago harbour. Variabilin was also identified in five other *Ircinia* species, and in ten species from the family Thorectidae.<sup>44</sup> The changeable nature of the activity, as the extract was purified, was shown to result from auto-oxidation of the variabilin.<sup>45</sup>

The other two species, an ascidian *Aplidium gilvum* (originally identified as *Pseudodistoma aureum*) and a bryozoan *Margaretta barbata* were collected by diving and are wide-spread in the sub-tidal waters of the Kaikoura peninsula. The extracts of *A. gilvum* displayed antiviral and cytotoxic activity, whilst the extracts of *M. barbata* were solely cytotoxic. These three species were unidentified when this work commenced.

### Separation Techniques

Typically a frozen marine organism is about 90% water, 3% salt and up to 0.3% soluble organic material. One of the major isolation problems with any extract is, therefore, the separation of the organic material from the water and salt. If the sample can be dried and then extracted with organic solvents there will be little problem with salt, but extraction of the wet sample is usually the only option, as air drying exposes the sample to oxidation and to micro-biological and chemical decomposition, and freeze drying using laboratory scale apparatus is impractical for anything more than a few hundred grams. Also polar solvents may be required to extract the compounds of interest.

Wet extraction, using methanol and a less polar solvent such as dichloromethane or toluene, has been used for most of the work described here. This method produces an extract which ranges in polarity from amino acids and oligopeptides to lipids and contains about 90% salt. The separation efficiency of the first purification step is of crucial importance. It is necessary to remove the salt, and, if

---

44 Perry, N.B., Battershill, C.N., Blunt, J.W., Fenwick, G.D., Munro, M.H.G. and Bergquist, P.R., *Biochem. Syst. Ecol.*, **15**, 373 (1987).

45 Barrow, C., PhD Thesis, (University of Canterbury : 1988).



possible, separate the organic compounds, by their polarity, into non-overlapping groups.

Liquid/liquid partitioning has been the mostly commonly used method. This technique requires no special equipment, and may be readily applied to large scale extracts. The system described by Kupchan<sup>46</sup> was the basis for the schemes used in the initial separations described in this section. Although liquid/liquid partitioning schemes may be optimised for a particular extract, such as that used by Pettit in the isolation of the bryostatins,<sup>47</sup> these multi-solvent methods often result in the biological activity being spread over several fractions.

A method with greater separation efficiency than liquid/liquid partitioning, that required no special equipment and that could be used for large scale extracts was therefore sought. Gel permeation chromatography permits the separation of a wide range of compounds, but is limited in sample capacity. Normal-phase materials and silica gel chromatography are not suited to polar compounds and ion-exchange chromatography is too specific to be used in the initial separation.

Reverse-phase chromatography is well suited to separating a wide range of sample polarities, but, in his review of the available techniques, Shimizu<sup>48</sup> dismissed this method because of its low sample capacity and the expense of commercially prepared columns. However it was found that very high loadings of crude extracts could be efficiently partitioned using reverse-phase supports in a nitrogen pressurised flash chromatography column.<sup>49</sup> Although commercial columns and reverse-phase materials are expensive, they may be readily and cheaply prepared. The method for making the support, adapted from that described by Evans *et al*,<sup>50</sup>

---

46 Kupchan, S.M., Britton, R.W., Ziegler, M.F. and Sigel, C.W., *J. Org. Chem.*, **38**, 178 (1973).

47 Pettit, G.R., Kamano, Y., Aoyagi, R., Herald, C.L., Doubek, D.L., Schmidt, J.M. and Rudloe, J.J., *Tetrahedron*, **41**, 985 (1985).

48 Shimizu, Y., *J. Nat. Prod.*, **48**, 223 (1985).

49 Blunt, J.W., Calder, V.L., Fenwick, G.D., Lake, R.J., McCombs, J.D., Munro, M.H.G. and Perry, N.B., *J. Nat. Prod.*, **50**, 290 (1987).

50 Evans, M.B., Dale, A.D. and Little, C.J., *Chromatographia*, **13**, 5 (1980).

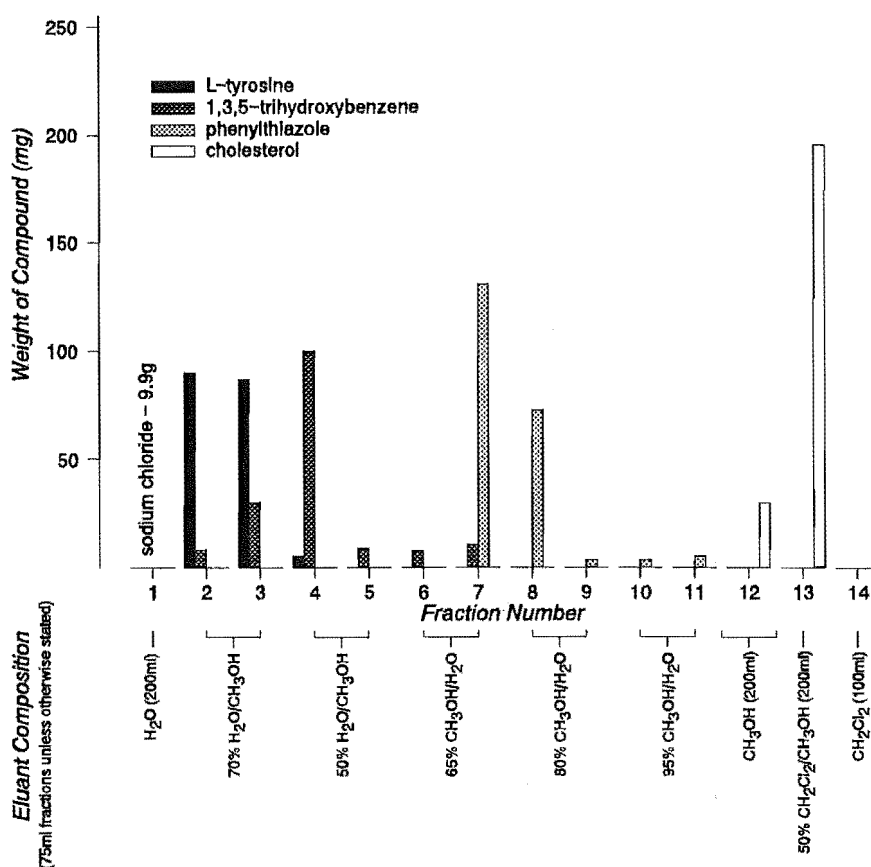
involved treating silica gel with the appropriate silyl trichloride, reacting the remaining chloride groups with methanol, and then finally "end-capping" any unreacted sites on the silica with trimethylsilylchloride. This procedure has also been successfully used to make octadecyl (C-18) and cyanopropyl TLC plates.

The method which was developed over a period of time, is as follows. The column is first packed using a methanol or chloroform slurry, then equilibrated with water. The sample may be prepared in two ways. A water/methanol/dichloromethane solution may be slurried with the reverse-phase material, then taken to dryness, *in vacuo*, to yield a powder which is then packed onto the column. A more convenient method is to make a water/dichloromethane solution/suspension of the sample, which is then slurried with the pre-wetted reverse-phase material. The dichloromethane is removed, coating the organic soluble components onto the support, to yield an aqueous slurry which is then packed onto the column. The sample should be prepared with at least an equal weight of reverse-phase packing.

The column is typically eluted with a steep stepped gradient from water through a range of water/methanol mixtures to methanol, then finally to dichloromethane. This method usually results in a clean partitioning of the mixture in terms of polarity. If care is taken, all the salt will generally elute in the first fraction, followed by the ionic, organic compounds. The polar compounds are eluted by solvent mixtures of 70 - 90% methanol/water, and the sterols, lipids and other non-polar compounds are eluted by dichloromethane or methanol/dichloromethane mixtures. Sample loadings of up to one-fifth of the weight of the material in the column were successfully used in the isolation of the active compounds from *Tedania* species (described in the next section).

The efficiency of the reverse-phase technique was tested using a mixture of compounds of widely varying polarity. L-tyrosine, 1,3,5-trihydroxybenzene, cholesterol and phenylthiazole (200mg each) were mixed with sodium chloride (10.2g). The test sample was dissolved in a water/methanol/dichloromethane mixture and coated onto C-18 support by removal of the solvent. The powder was packed onto a C-18 column (100g), which was then eluted with a steep, stepped

Virtually all of the salt was eluted in the first fraction. With the exception of fraction three, the compounds were recovered in high purity. This clean separation of compounds, on the basis of their polarity, was also obtained with the natural



product extracts that were processed in this manner.

Undoubtedly the success of the reverse-phase technique is a result of the large variation in the polarity of the sample compounds. Although the total loading of the column is high, only about ten percent of a marine extract is organic in nature and only a small portion that organic material will be soluble for any given eluant composition. The separation in this situation could perhaps be viewed as a liquid/solid partitioning process occurring in concert with the reverse-phase chromatography.

It is difficult to overestimate the importance of this technique in dealing with the type of extracts encountered in natural products work, especially where the active

liquid/solid partitioning process occurring in concert with the reverse-phase chromatography.

It is difficult to overestimate the importance of this technique in dealing with the type of extracts encountered in natural products work, especially where the active compounds are relatively polar and large quantities of extract must be processed to concentrate compounds which are present at low levels. The technique can deal with the full range of compounds in a total extract, in a manner that avoids the decomposition problems associated with chromatographic materials such as silica gel. In addition almost none of the sample is irreversibly absorbed, hence the column may be re-used many times. The only special equipment required is a gas pressurised flash column.

The reverse-phase technique was developed at the same time that the initial evaluation of the extracts of *A. gilvum* and *M. barbata* was taking place. In both cases reverse-phase chromatography of the crude extracts proved to be more convenient than conventional liquid/liquid extraction techniques.

### ***Aplidium gilvum***

The antiviral assays of the extracts of a number of collections of *A. gilvum* gave varying results, but most displayed activity against both HSV-1 and PV1, with some degree of cytotoxicity.

*A. gilvum* (2.1kg) was homogenised and extracted repeatedly with methanol/toluene and methanol/water mixtures, to yield twenty grams of crude extract.

The initial fractionation was by liquid/liquid extraction. An aliquot (0.6g) of the extract was dissolved/suspended in 90% methanol/water. The sample was then extracted with pet. ether. The composition of the methanol partition was adjusted to 70% methanol by the addition of water, and re-extracted with dichloromethane. Further water was added to the methanol fraction to bring its composition to 30% methanol, and it was re-extracted with ethyl acetate. Finally the methanol was

removed, *in vacuo*, from the methanol/water fraction. The aqueous fraction was then extracted with butan-1-ol. For the antiviral assay the volume of each of the partitions was adjusted so that concentrations were equivalent to those in the initial screening assays. Although activity was found in three of the five fractions, the most intense antiviral activity was found in the butan-1-ol fraction.

Preparative TLC was used to test the stability of the active components on silica gel. A band of the butan-1-ol fraction was applied to a commercial TLC plate and eluted with 50% methanol/dichloromethane. The plate was divided into three bands, and assays of the recovered material indicated that the active compounds were in the fraction above  $R_f$  0.49. The active fraction showed strong cytotoxicity as well as antiviral activity.

A portion of the crude extract (880mg) was applied to a silica gel column and eluted with a stepped solvent gradient from pet. ether through ether and ethyl acetate to methanol. The twenty-five fractions collected were combined on the basis of TLC into seven fractions. The antiviral and cytotoxic activity was located in fractions six and seven, which were eluted with methanol/ethyl acetate mixtures. TLC analysis of fraction six (16mg) showed the presence of at least seven compounds. The final fraction, eluted with methanol, displayed only cytotoxic activity.

In an effort to obtain more material, the remainder of the crude extract was partitioned, by liquid/liquid extraction, between ethyl acetate, butan-1-ol and water. This time the ethyl acetate and aqueous fractions were found to be active, and not the butan-1-ol fraction. The three fractions were re-combined. A methanol/water solution/suspension of the combined fractions was coated onto some C-8 reverse-phase material by removing the solvent from a slurry, under reduced pressure. The extract was loaded onto the flash column and eluted with a steep, stepped gradient from water through to methanol, then dichloromethane.

The forty-four fractions collected were combined into six, on the basis of reverse-phase TLC. Fractions four to six, eluted with methanol or methanol/dichloromethane were found to have only cytotoxic activity.

***Margaretta barbata***

*M. barbata* occurs widely on the rocky coastline around Kaikoura. Extracts of a number of samples of the bryozoan exhibited strong cytotoxicity in the antiviral assay.

A sample of *M. barbata* (2.1kg wet weight) was extracted with propan-2-ol, dichloromethane and water to yield a dark brown malodorous tar (50g).

Liquid/liquid partitioning of a portion of this extract (1.5g), between dichloromethane, ethyl acetate, butan-1-ol and water failed to separate the active components from the salt-containing aqueous fraction.

An aliquot of the crude extract (1.2g) was chromatographed on silica gel. The twenty-six fractions which were collected were combined into eight fractions, on the basis of silica gel TLC analysis. Antiviral and cytotoxic activity was found in six fractions, but the first, eluted with pet. ether, and last fractions, eluted with methanol, were by far the most active. All the fractions were shown to be complex mixtures by silica gel TLC. Normal-phase HPLC of fraction one, from the previous step, using a cyanopropyl semi-preparative scale column, yielded four fractions which displayed no antiviral or cytotoxic activity.

Since it appeared that the active compounds were polar in nature a new approach was tried. A portion of the crude extract (2.1g) was partitioned between water and dichloromethane. The aqueous fraction, which exhibited only antiviral activity, was freeze dried. The residue was washed with dry methanol, in a partially successful attempt to separate the organic material from the salt. The remaining residue was dissolved in water for assay. The active compounds were located in the methanol soluble fraction; however this fraction was still largely salt.

The methanol fraction (1g) was chromatographed on a C-8 reverse-phase flash column. The thirty-seven fractions which were eluted with a stepped solvent gradient, from 50% methanol/water to methanol then dichloromethane, were combined into six fractions after analysis by reverse-phase HPLC. The cytotoxic activity was found to have been successfully concentrated in the fractions which

were eluted with methanol. TLC analysis of the most active fraction indicated that it was a complex mixture. The results of the reverse-phase separation were sufficiently encouraging to warrant attempting fractionation of a crude extract in this way.

To ensure that the cytotoxicity shown by *M. barbata* was not due to epibionts, a sample was carefully cleaned of the sponges which typically colonise the surface of this bryozoan. The sample (850g) was then homogenised and extracted with methanol/toluene and methanol/water. Removal of the solvents, under reduced pressure, afforded twenty-five grams of extract.

Half the extract was coated onto C-8 material by removing the solvents from a slurry, then loaded on the C-8 flash column. Forty-eight fractions were eluted with a solvent gradient from water through methanol to dichloromethane. The antiviral assay revealed cytotoxic activity in the fractions eluted with water or 5% methanol/water and in the fractions eluted with 95% and 90% methanol/water.

Further reverse-phase chromatography of the lipophilic fractions was attempted using a Lobar MPLC column and a solvent gradient from 70% methanol/water to methanol, but a similar level of activity was found in all the fractions collected.

## Conclusion

Although the results of the preliminary studies on *A. gilvum* and *M. barbata* showed promise it was decided that work on these species should be discontinued for several reasons. *A. gilvum* was initially chosen because of its potential as a source of antiviral compounds, however the antiviral activity seemed to vary as the purification proceeded and was accompanied by increasing levels of cytotoxicity. The use of a reverse-phase column as the first step in the fractionation of the *M. barbata* extract proved efficient. However, the extract of a newly collected sponge species, *Tedania* n. sp. B, was found to possess strong antiviral activity and no cytotoxicity. Since the isolation of antiviral substances was the primary goal at this stage of the project, the extract of this sponge was given the highest priority. The

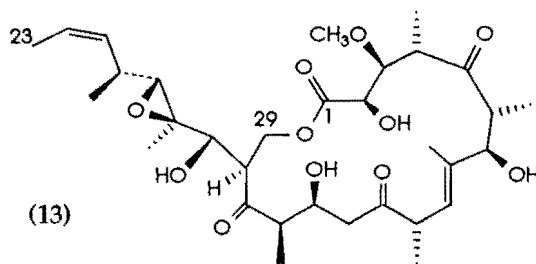
results of the study of the *Tedania* sponges is described in the next section of this thesis.



### Compounds from *Tedania* Species

---

The unidentified brown amorphous sponges, designated PML1-6, 5P2-24 and 5P3-1, collected from the channel near Portobello in Otago Harbour, were chosen for investigation. The screening extracts showed a high degree of antiviral activity, with little or slight cytotoxicity, and sponge was readily available. Three antiviral compounds were eventually concentrated and tentatively identified as the known compound tedanolide (13) and two related compounds.



The sponges, *Tedania* species, were not identified<sup>51</sup> until the isolation work was well advanced. At that point a review of the available spectroscopic and bio-assay data from the partially purified extract failed to show any evidence of the compounds which had been previously reported from sponges of the *Tedania* genus.

The genus *Tedania* is one of two in the family Tedaniidae Ridley and Dendy.<sup>52</sup> This family is a member of the Poecilosclerida Topsent which is the largest and most diverse order of the Demospongiae.<sup>53</sup> *Tedania* species are widely distributed around the New Zealand coastline, with samples having been collected from

---

51 Identification by P.R. Bergquist.

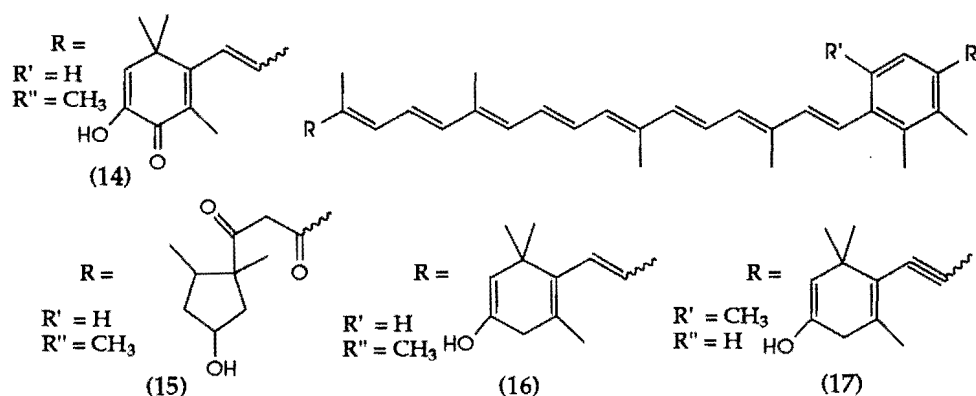
52 Bergquist, P.R. and Fromont, P.J., NZOI Memoir, 96 (1988).

53 Bergquist, P.R., *Sponges* (Hutchinson : London 1978).

Leigh, north of Auckland, Wellington, Kaikoura, Akaroa, Stewart Island, Milford Sound and the Auckland Islands (see map on page 15).

### Compounds Previously Reported from *Tedania* Species

A number of compounds have been reported from sponges of the *Tedania* genus. The aromatic carotenoid tedanin (14) was isolated from *Tedania digitata* (Schmitz)<sup>54</sup> and subsequently synthesised. A further three carotenoids, (15),<sup>55</sup> (16)<sup>56</sup> and (17),<sup>57</sup>



were isolated from the Japanese samples of *Tedania digitata*. The carotenoid (14) was also found in the sponge *Clathria frondifera*,<sup>58</sup> and had been previously reported as occurring in the sponge *Trikentrion helium*.<sup>59</sup>

54 Okukado, N., *Bull. Chem. Soc. Jap.* **48**, 1061 (1975).

55 Tanaka, Y., Fujita, Y. and Katayama, T., *Bull. Jap. Soc. Sci. Fish.*, **43**, 6, 767 (1977).

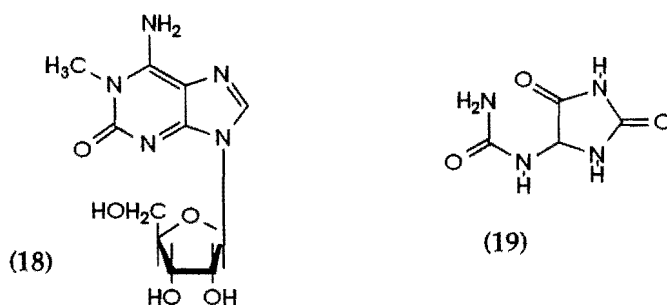
56 Tanaka, Y., Fujita, Y. and Katayama, T., *Bull. Jap. Soc. Sci. Fish.*, **43**, 6, 761 (1977).

57 Tanaka, Y. and Katayama, T., *Bull. Jap. Soc. Sci. Fish.*, **43**, 6, 381 (1980).

58 Shimada, A., Ezaki, Y., Inanaga, J., Katsuki, T. and Yamaguchi, M., *Tetrahedron. Lett.* **22**, 773 (1981).

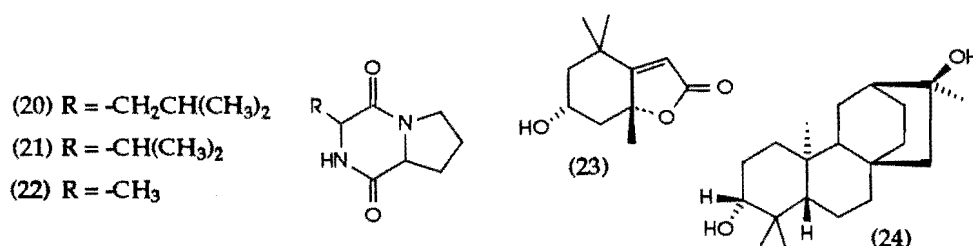
59 Aguilar-Martinez, M. and Liaaen-Jensen, S., *Acta Chem. Scand.*, **B28**, 1247 (1974).

Ethanollic extracts of *Tedania digitata*, collected in Australia, were found to have muscle relaxant, anti-inflammatory and other pharmacological activities.<sup>60,61</sup> The methylated nucleoside 1-methylisoguanosine (18) and allantoin (19) were isolated



from *Tedania digitata* by a combination of diafiltration, gel permeation and ion-exchange techniques.

The Caribbean species *Tedania ignis* is known as the fire sponge, because of its ability to cause contact dermatitis.<sup>62</sup> However, it was the cytotoxicity and *in vivo* antitumour activity of extracts of this sponge that aroused the interest of Schmitz's group.



In one study<sup>63</sup> several marginally cytotoxic, or inactive, compounds were reported. The diketopiperazines (20) - (22),  $\delta$ -valerolactam and *p*-hydroxybenzaldehyde were isolated from a propan-2-ol extract of the sponge, by a combination of gel

60 Quinn, R.J., Gregson, R.P., Cook, A.F. and Bartlett, R.T., *Tetrahedron. Lett.*, **21**, 567 (1980).

61 Cook, A.F., Bartlett, R.T., Gregson, R.P. and Quinn, R.J., *J. Org. Chem.*, **45**, 4020 (1980).

62 de Laubenfels, M.W., *Trans. Zool. Soc. London*, **27**, 1, 1 (1950).

63 Schmitz, F.J., Vanderah, D.J., Hollenbeak, K.H., Enwall, C.E.L., Gopichand, Y., Sengupta, P.K., Hossain, M.B. and van der Helm, D., *J. Org. Chem.*, **48**, 3941 (1981).

permeation and silica gel chromatography. The diketopiperazines had previously been isolated from fungi and plants, and there was some discussion as to whether (20) - (22) were sponge metabolites, or of microbial origin. Two further compounds were isolated using silica gel and DEAE-cellulose chromatography. The first was the previously reported<sup>64</sup> epiloliolide (23), and the second a new mildly cytotoxic atisane derivative (24). Also identified were 3-(octadecyloxy)-propan-1,2-diol and 3-(hexadecyloxy)-propan-1,2-diol. More recently, the three diketopiperazines (20)-(22) were obtained from extracts of cultures of a marine bacterium, a *Micrococcus* species. The bacterium was isolated from samples of *T. ignis*, collected in Bermuda.<sup>65</sup>

A second report<sup>66</sup> described the isolation of a potent cytotoxic macrolide, tedanolide (13), from a methanol/chloroform extract of *T. ignis*. The pure compound was obtained by a combination of liquid/liquid extraction, gel permeation and silica gel chromatography and reverse-phase HPLC, with a yield of one part per million. The structure, including the absolute configuration, was solved by single crystal X-ray crystallography.

Tedanolide was reported to have intense *in vitro* cytotoxic activity with an ED<sub>50</sub> for the PS (P388 cell line) and KB systems of 16pg/ml and 250pg/ml respectively. No antiviral activity was reported.

A water soluble toxin was isolated from *T. ignis*, collected from mangrove swamps in Venezuela.<sup>67</sup> Subsequent studies provided evidence that the toxin was accumulated from dinoflagellate which was prevalent in the mangrove forests.<sup>68</sup>

---

64 Horii,Z., Yagami,T., Ito,M. and Hanaoka,M., *Chem. Pharm. Bull.*, **16**, 848 (1968).

65 Stierle,A.C., Cardellina,J.H. and Singleton,F.L., *Experientia*, **44**, 1021 (1988).

66 Schmitz,F.J., Gunasekera,S.P., Yalamanchili,G., Hossain,M.B. and van der Helm,D., *J. Am. Chem. Soc.*, **106**, 7251 (1984).

67 Sevcik,C. and Barboza,C.A., *Toxicon*, **21**, 191 (1983).

68 Mebs,D., *Toxicon*, **22**, 821 (1984); Mijares,A.J., Sevcik,C., Barboza,C.A. and Saavedra,J.A., *Toxicon*, **23**, 221 (1985); Sevcik,C., Mijares,A.J. and Saavedra,J.A., *Toxicon*, **24**, 413 (1986).

Like *T. ignis*, the *Tedania* species collected in New Zealand provoked a marked dermatological reaction. *T. battershilli* was the most powerful irritant, and some collectors were more susceptible than others.

### ***Sterols from Tedania species.***

---

The lipid fraction of extracts of *Tedania* species was analysed by capillary GC in the hope of distinguishing the sponges which displayed markedly different surface morphologies. Attempts to identify the components for comparison with published work were unsuccessful.<sup>69</sup>

Nine samples of *Tedania* species were cut from single sponge colonies to ensure purity. Extracts of these sponges were compared with a previously collected sample of Portobello *T. connectens*. The crude extracts were derivatised with trimethylsilylimidazole (TMSIM). In early trials the sterol fraction was isolated from the crude extract before derivatisation, but the chromatograms obtained from the crude extracts contained no additional peaks, so the purification step was eliminated. TMSIM was chosen as the derivatising agent because it is a powerful silyl donor towards alcohols and carboxylic acids, but has few side reactions.<sup>70</sup>

The chromatograms obtained were the same to within experimental error. In spite of repeated attempts, the only compound for which a GCMS mass spectrum could be obtained was cholesterol, the major component. The samples were subsequently identified as *T. connectens*, except for one which was *T. diversirhaphidiophora*. Unfortunately these results were not sufficiently encouraging to warrant further investigation.

---

69 Bergquist, P.R., Hofheinz, W. and Oesterhelt, G., *Biochem. Syst. and Ecol.*, **8**, 423 (1980).

70 Horning, E.C. and Thenot, J.-P., *Anal. Lett.*, **5**, 21 (1972), and Horning, E.C., *et al*, *Biochem. and Biophys. Acta*, **148**, 597 (1967).

### ***Isolation of the Antiviral Components of the Tedania Genus***

---

The active compounds were isolated by a combination of reverse-phase, silica gel and gel permeation chromatography. An outline of the separation process is shown in the "separation tree" charts on the succeeding pages. Where a sample gave a positive antiviral assay result, at several concentrations, the lowest dose that gave a "whole well" response is shown. If a "whole well" response was not obtained the chart shows the result for the highest concentration assayed. The concentration is expressed as  $\mu\text{g}$  of sample per assay disk. Occasionally the samples were assayed at an unknown concentration (e.g. column effluent), in which case the concentration is a volume per disk. Some steps in the separation trees have mass balance discrepancies. These mostly arise from the fact that individual fractions were not weighed as accurately as the combined fractions. Some differences may be attributable to irreversible adsorption of material on the columns.

The separation tree charts are also in the fold-out section at the back of this thesis.

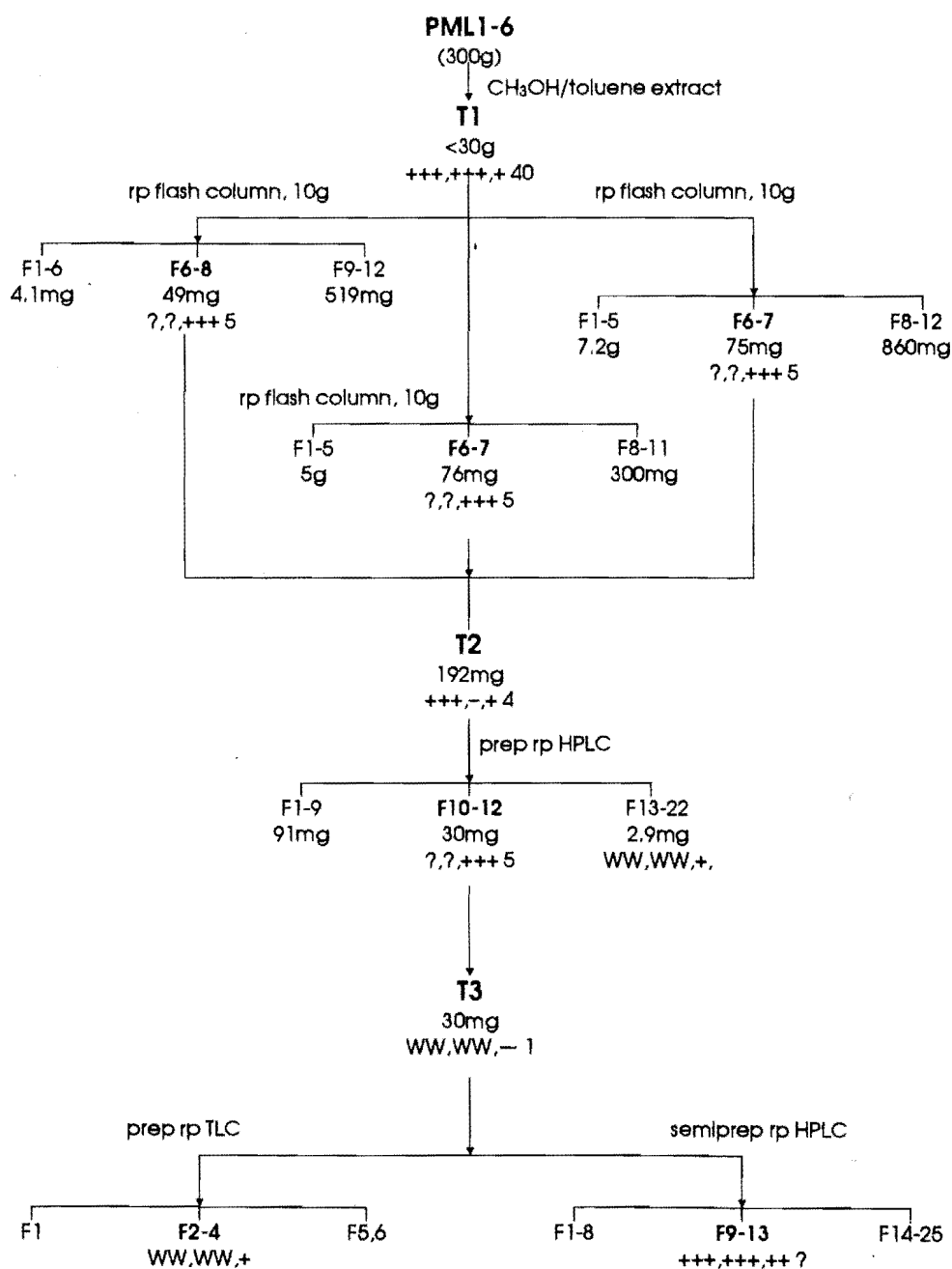
The isolation of the antiviral components is discussed first. This is followed by a discussion of the evidence for the identity of these compounds.

#### ***Extraction and Trial Chromatography of PML1-6: Extract A***

Exploratory chromatography was conducted on a toluene/methanol/water extract of the sponge sample PML1-6 (300g wet weight). The extract was made by homogenising the sponge and repeatedly extracting with methanol/toluene then methanol/water mixtures. Removal of the solvents afforded a brown malodorous tar.

The tar was chromatographed, in three ten gram aliquots, on a C-8 reverse-phase open column, using a steep stepped gradient from water through methanol to dichloromethane. Each fraction was submitted for antiviral assay and the active fractions from the three columns were combined to yield a green-brown gum (sample T2, 192mg). The entire amount of sample T2 was coated onto a small

### Separation Tree for the PML1-6 Extract



amount of C-8 support (10g) by removing the solvent from a slurry, *in vacuo*. The coated C-8 material was packed into a pre-column which was attached to the C-8 MPLC column. The column was eluted with a stepped methanol/water gradient from 50% to 90% methanol. The twenty-five fractions were assayed. The antiviral activity was located in fractions ten to twelve, which were combined as sample T3.

An aliquot of T3 was applied as a band to a reverse phase TLC plate. The plate was developed with 70% methanol/water, and visualisation of the edge, with phosphomolybdic acid, revealed at least six bands at medium to low  $R_f$ . The plate was cut longitudinally. One half of the plate was divided into six bands corresponding to the spots and the adsorbent scraped off. The scrapings were washed with methanol and the solutions were assayed. The fractions between  $R_f$  0.19 and 0.53 were found to be active. The other half of the plate was applied, adsorbent-side down, to a 40mm well infected with HSV, in an attempt to obtain a bioautograph. The procedure was unsuccessful.

Another aliquot of T3 was chromatographed on a reverse-phase C-18 semi-preparative column with 70% methanol/water. The UV-detected (210nm) chromatogram was a poorly resolved envelope, but the antiviral activity was confined to just five (fractions 9 to 13) of the twenty-five (2.5ml) fractions collected.

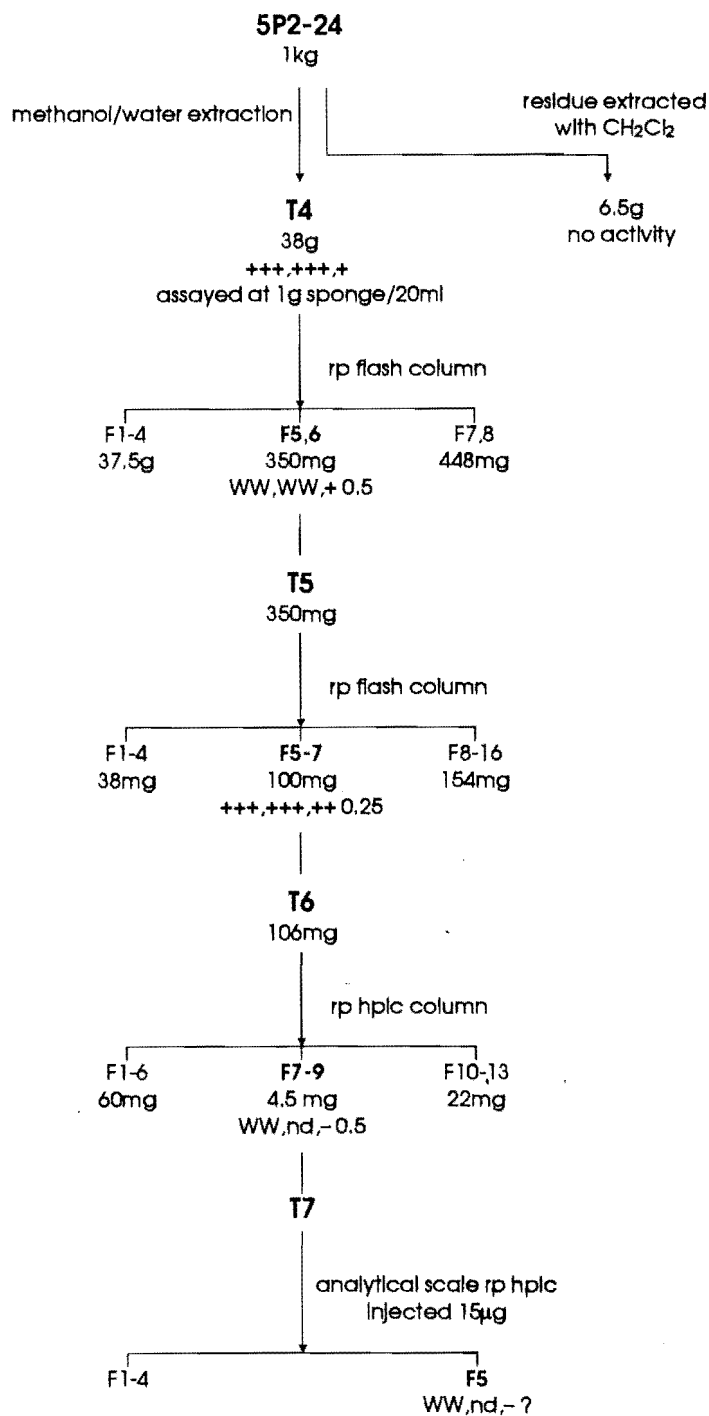
The results of this preliminary study suggested that the antiviral compounds in the extract were sufficiently stable and chromatographically well enough behaved to justify work on a larger quantity of extract.

#### **Extraction of 5P2-24: Extract B**

A further twenty-five kilograms of *T. connectens* was collected from the channel near Portobello, Otago Harbour. One kilogram of homogenised sponge (collection number 5P2-24) was extracted several times with 70% methanol/water, methanol and dichloromethane. Antiviral assays indicated that the active compounds were contained entirely in the methanol and aqueous extracts, which were combined (sample T4).



### Separation Tree for 1kg Extract of 5P2-24



Reverse-phase flash chromatography was used for the initial purification. An aqueous solution/suspension of the total amount of T4 (38g), was slurried with a quantity of C-18 adsorbent, and packed onto the column. A steep stepped solvent gradient from water through methanol was used to elute eight fractions. The bulk of the antiviral activity was located in the fifth fraction, eluted with 80% methanol/water. This pale green fraction had a distinctive fishy odour. Reverse-phase TLC analysis indicated the presence of more than six compounds.

The active fractions (five and six, 350mg) were combined, as T5, and re-chromatographed by reverse-phase flash chromatography, eluting with water/methanol mixtures from 50% to 100% methanol, then stripping with 20% methanol/dichloromethane. Assays indicated that the antiviral material had been eluted with 70% methanol/water. These fractions were then combined as sample T6 (106mg).

Sample T6 could not be completely redissolved in methanol or methanol/water mixtures. The 88mg of soluble material was purified by semi-preparative scale reverse-phase HPLC. Thirteen fractions were collected on a volume basis, and the antiviral activity was located in three consecutive fractions, T7, totalling 4.5mg. Further purification was attempted using a C-18 analytical scale HPLC column, but it was clear that more material would be required.

It was not clear what proportion of the 4.5mg obtained in the last step was the active component. The assumption was made that the biological activity of the antiviral compounds was comparable to that of the strongly active mycalamides A (P388  $IC_{50}$   $3.0 \pm 1.3$  ng/ml)<sup>71</sup> and B (P388  $IC_{50}$   $0.7 \pm 0.3$  ng/ml)<sup>72</sup> On this basis it was estimated that about the active constituent was present at about about one part in fifty. It was hoped that the large scale isolation described in the next section might yield several milligrams.

---

71 Perry,N.B., Blunt,J.W., Munro,M.H.G. and Pannell,L.K., *J. Am. Chem. Soc.*, **110**, 4850 (1988).

72 Perry,N.B., Blunt,J.W., Munro,M.H.G. and Thompson,A.M., submitted to *J. Org. Chem.*

***Large Scale Extraction of 5P2-24 and 5P3-1: Extract C***

A further twenty-four kilograms of frozen sponge (5P2-24 and 5P3-1) was available for extraction. Scaling up the wet extraction process was expected to yield almost a kilogram of extract, the majority of which was salt. Earlier results suggested that the antiviral material could be completely extracted using only methanol as a solvent, hence the desalting problem could have been reduced considerably by extracting dry sponge. A trial with a kilogram of sponge (133g dry weight) confirmed the feasibility of this approach. Unfortunately freeze-drying facilities capable of handling the larger quantity of sponge could not be found, and it was therefore decided to extract the frozen material.

The remainder of the frozen sponge was homogenised and repeatedly extracted with methanol and 90% methanol/water, in two batches. A total of two litres of solvent per kilogram (approximately 130g dry weight) of wet sponge were used. The filtrate from the final extraction cycle of the first batch was assayed separately to ensure that all the antiviral material had been removed. One kilogram of a brown malodorous tar was obtained, after the solvents had been removed from the combined extracts (T8) in a cyclone evaporator, a device for removing large quantities of solvent at low temperatures (<35°C)<sup>73</sup>

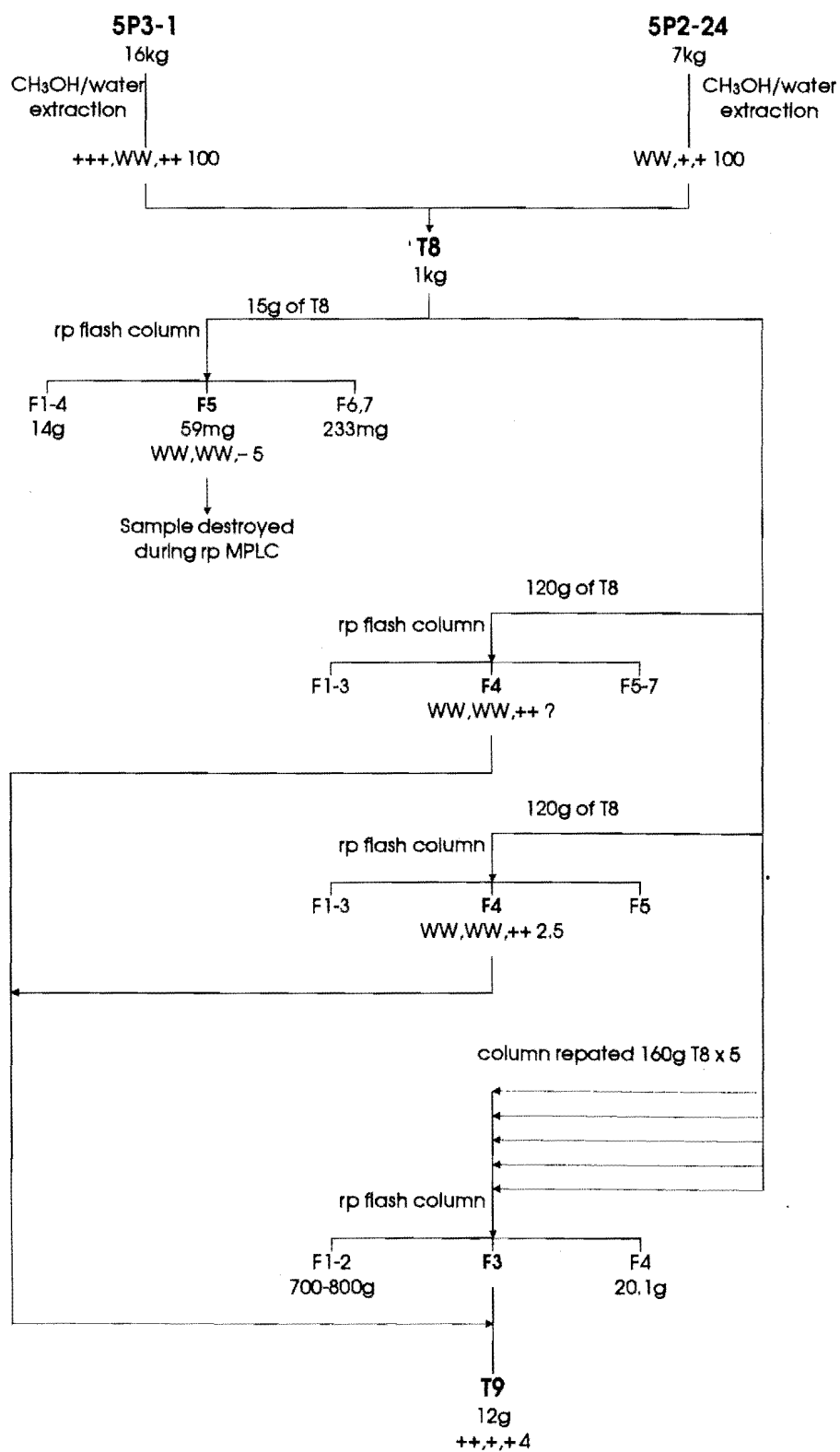
A trial separation of T8 (15g) was made using C-18 flash chromatography with water/methanol mixtures. As expected the active material (59mg) was eluted by 80% methanol/water, and had the previously noted sharp fishy odour. This and many of the active fractions from subsequent purification steps required the addition of a few drops of octan-1-ol to suppress foaming during solvent evaporation.

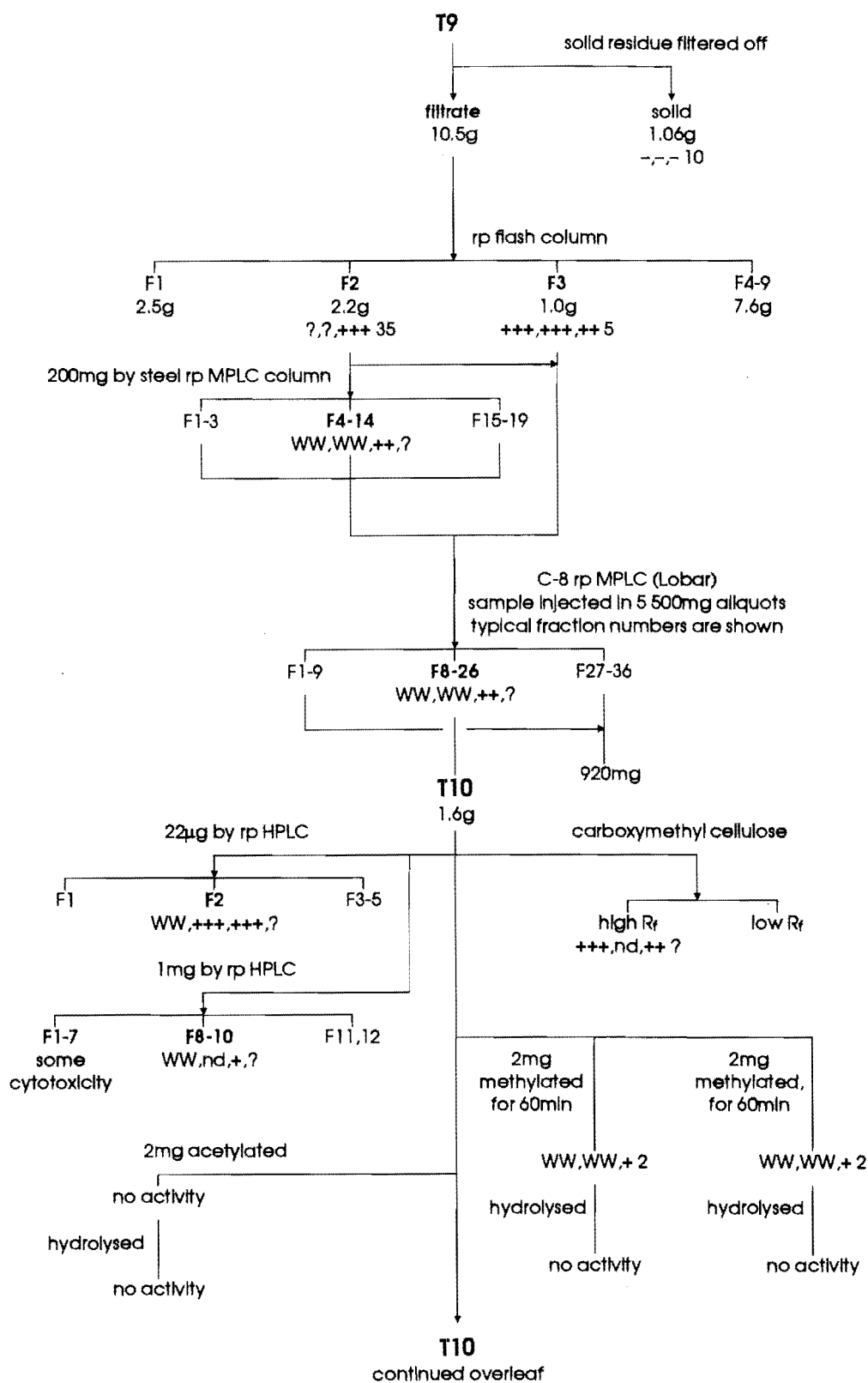
Using a steep stepped gradient derived from the trial column, the remainder of the crude extract was chromatographed in seven aliquots (up to 160g at a time on 400g of C-18). The antiviral material was eluted from each column by the 80%

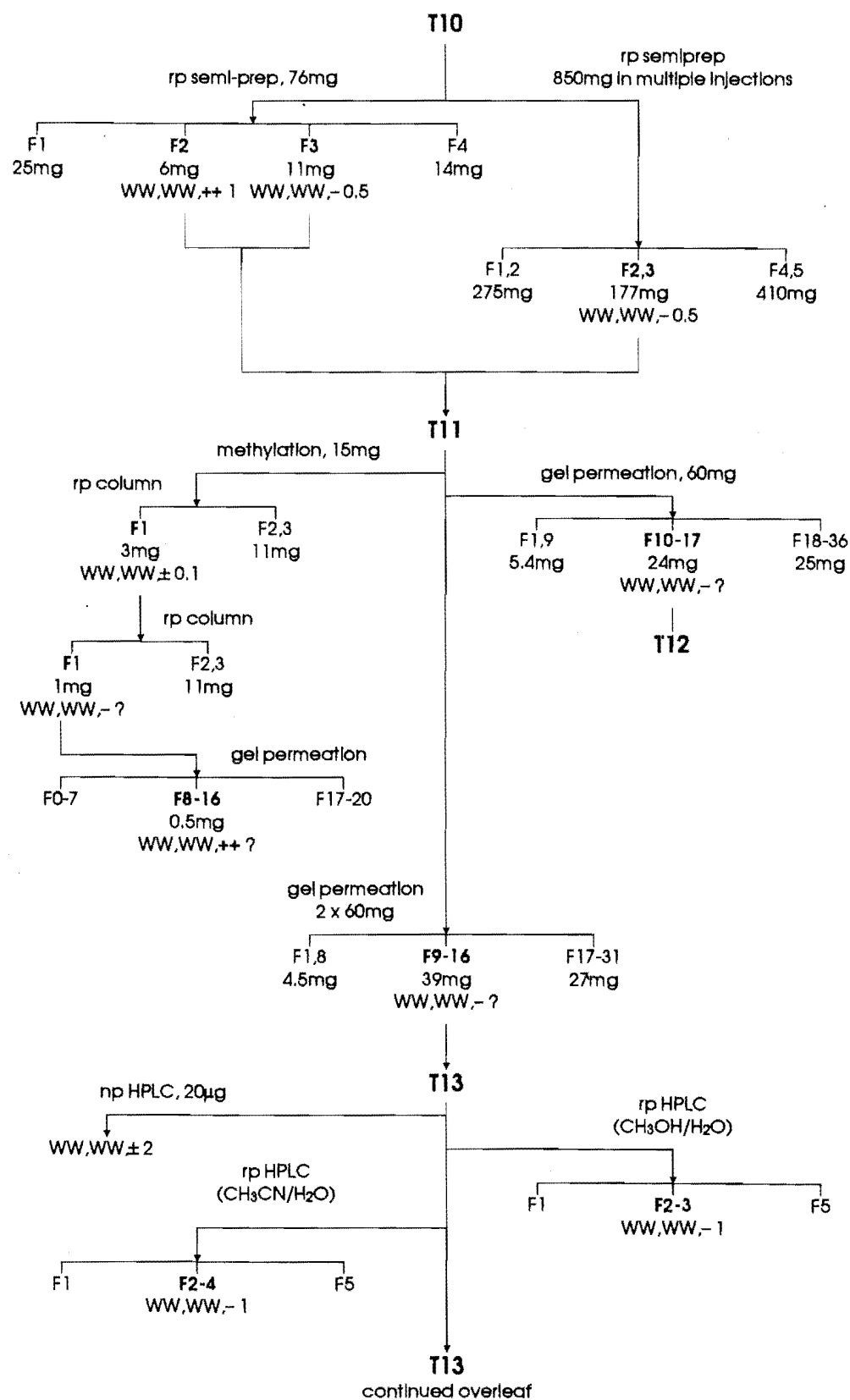
---

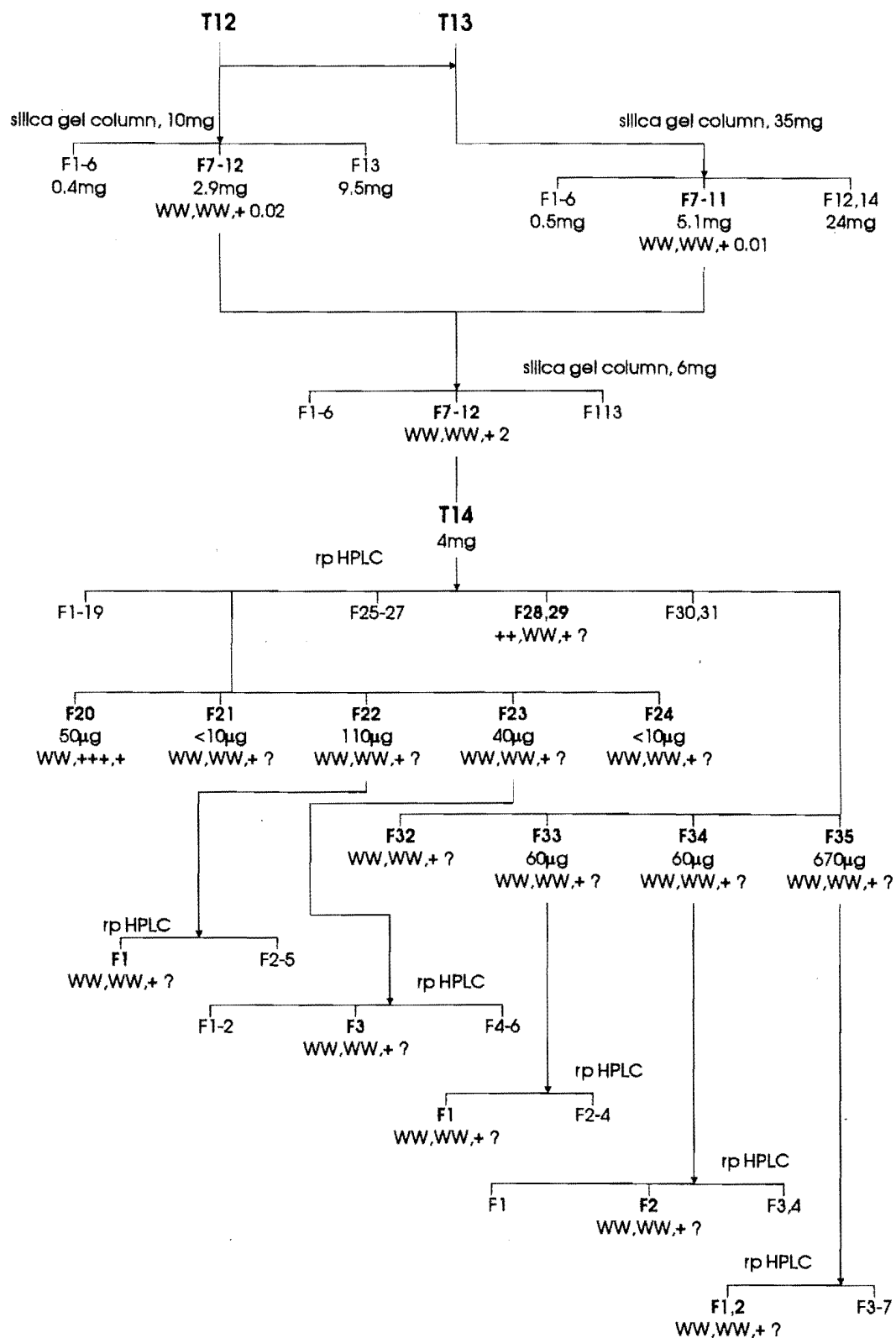
73 Schmitz, F.J., personal communication.

### Separation Trees for the Extract of 5P2-24 and 5P3-1









methanol/water mixture and combined as sample T9. Solvents were removed under reduced pressure using a cyclone evaporator, to yield a green odiferous gum. On standing at 4°C this fraction threw down a fine white precipitate (1.06g) which was relatively insoluble in water, methanol and dichloromethane. The solid, which was partially soluble in 0.05% TFA/water, exhibited no antiviral activity and had a relatively featureless <sup>1</sup>H-NMR spectrum. It was not investigated further due to the lack of activity and the expense of suitable NMR solvents.

The filtrate (10.5g) was re-chromatographed on C-18 reverse-phase adsorbent. Nine fractions were collected, using a stepped solvent gradient from 50% methanol/water to methanol then 0.05% TFA/methanol. The fractions two and three, eluted with 65% methanol/water (3.2g), were found to be active.

C-18 support was prepared from 20-30μ silica gel and packed into a steel column as a carbon tetrachloride slurry to ensure an even bed. The column was washed and equilibrated with 70% methanol/water. Separation a portion of fraction two, from the previous step, was attempted with isocratic elution. The UV chromatogram (205nm) was an almost featureless envelope and the antiviral assay indicated only poor separation was obtained. The active fractions from this separation were combined with the remainder of the active fractions from the previous step.

An aliquot (500mg) of the combined material was chromatographed using a commercial C-8 MPLC column, with 60% methanol/water as the eluant. The results of the separation were sufficiently good to warrant chromatographing the rest of the active material in this system. The active fractions from the five runs were combined as sample T10 (1.6g).

The small weight reduction in the previous step (3.2g to 1.6g) indicated that C-18 MPLC was unlikely to be of further use. Several small scale experiments were carried out in an attempt to find a suitable technique to further purify the active components.



Cation-exchange chromatography, of T10, was attempted using carboxymethyl cellulose TLC paper. The eluant was an aqueous acetic acid / ammonia buffer (pH 4), mixed with methanol (1+1). Two spots were visible on the chromatogram. The spot at high  $R_f$  was observed with iodine, ninhydrin and phosphomolybdic acid visualising reagents, while the low  $R_f$  spot was only visible with ninhydrin. It was found that only the high  $R_f$  spot had antiviral activity. This result suggested that the active compounds were either neutral or acidic. No anion-exchange materials were available.

The second approach tried was chemical modification. Acetylation and methylation are frequently used in natural products separations in the hope of changing the chromatographic properties of some of the components in an otherwise chromatographically homogenous mixture.

Diazomethane, which usually reacts only with carboxylic acids, was used to methylate two aliquots (2mg each) of T10. One was quenched after one minute and the other after one hour. The reaction mixtures, which still exhibited antiviral activity, were analysed by silica gel TLC and new high  $R_f$  spots were observed. The reaction mixtures were then hydrolysed. The hydrolysis products had no antiviral activity and the only spot observed in the TLC analysis did not correspond to any observed for T10.

Another aliquot was acetylated with acetic acid / pyridine. Once again several new spots, including some at high  $R_f$ , were observed, but the reaction products had no antiviral activity. Subsequent hydrolysis failed to restore any activity.

An initial trial, chromatographing an aliquot of T10 (22 $\mu$ g) on an analytical scale C-18 HPLC column and taking five fractions, yielded encouraging results. The separation was repeated with a new column and 62.5% methanol / water as the eluant. Twelve fractions were collected, of which three exhibited antiviral activity. The active fractions all had a characteristic sharp fishy odour.

Reverse-phase semiprep HPLC was chosen as the next separation step. Although the methylation experiments gave promising results, it was decided that chemical

modification was not appropriate when the chromatographic techniques had not been exhausted.

The conditions for this separation were established with an initial aliquot of T10 (76mg), which was chromatographed 7mg at a time. The fractions were selected to isolate groups of peaks in the UV chromatogram (210nm). The second and third fractions (total 17mg) were found to have antiviral activity. The remaining 850mg of sample T10 was chromatographed in many injections, with the fraction sizes slightly modified so that the active material was contained in one fraction. All the active material was combined as sample T11.

Another evaluation of the usefulness of methylation was made with a samples of T11. An aliquot of T11 (15mg) was methylated with diazomethane, then chromatographed on a C-18 open column with methanol/water mixtures. The antiviral material (3mg), eluted with 65% methanol/water was re-chromatographed using 60% methanol/water taking much smaller fractions.

A gel permeation column was prepared (100g Fractogel PGM2000) and approximate values for  $V_0$  and  $V_i$  established. The combined active fractions (1mg) were applied to the column, which was then eluted with methanol. After the void volume had been discarded, twenty-two 5ml fractions were collected. The antiviral activity was located in fractions eight to sixteen (0.5mg).

Although the process of methylation followed by chromatography appeared to work well it was once again decided that modification of the mixture of unknown compounds should not be tried while a number of chromatographic possibilities remained untried.

An aliquot of T11 (60mg) was applied to the column, which was then eluted with methanol. The void volume was discarded, and thirty-six fractions were collected. The antiviral material was combined as sample T12 (24mg). Another two portions of T11 (60mg each) were chromatographed using the same gel permeation conditions. The active fractions from these separations were combined to yield sample T13.

At this point a number of HPLC systems were explored. Two normal-phase solvents systems were evaluated using an analytical scale cyanopropyl column. Methanol/chloroform mixtures gave no retention of the UV absorbing material, while chloroform/pet. ether mixtures resulted in poor resolution and peak shapes. The effluent of an experiment run in pure chloroform (20 $\mu$ g injected) gave a positive antiviral assay, which indicated that the antiviral material was stable and could be eluted under normal-phase conditions.

C-18 reverse-phase chromatography with water/methanol and water/acetonitrile mixtures gave almost identical results. The UV chromatograms were poorly resolved envelopes in the region from one to six column volumes.

Another experiment in which T12 (10mg) was applied to a silica gel column, and eluted with methanol/dichloromethane mixtures, gave a more satisfactory separation. The remainder of samples T12 and T13 were combined (35mg) and chromatographed in a similar manner. The active fractions from these two columns were combined and re-chromatographed. The antiviral fractions were again combined, as sample T14 (4mg).

Sample T14 was chromatographed by repeatedly injecting 100 $\mu$ g aliquots onto a C-18 analytical column, and eluting with 65% methanol/water. The fractions were collected on a volumetric basis, but the size of the fractions was adjusted for experimental drift such that the fraction collected always remained in the same position relative to the peaks in the UV chromatogram. Several groups of fractions contained antiviral activity, which suggested that there was more than one antiviral compound in the mixture. The fractions from this separation step will be referred to as derivatives of T14, for example fraction one will be designated T14.1.

Fractions T14.21 through T14.24, and T14.32 through T14.35 were submitted for DCI and HR FAB mass spectrometry. Those fractions whose DCI spectra had strong high mass ions, T14.22, T14.23 and T14.33 to T14.35, were selected for further chromatography.

These five fractions were chromatographed using a new high quality C-18 analytical column with 50% acetonitrile/water. Fractions were collected so as to isolate peaks in the UV chromatograms (195nm). The UV chromatograms for each of these fractions are shown on the following page. The fractions containing antiviral activity are indicated. The masses of the antiviral fractions was estimated to be in the range of one to five micrograms. An acetonitrile/water solvent system was chosen for two reasons. Firstly it appeared to give better peak shapes than the methanol/water system, and secondly it enabled the UV detector to be operated at a shorter wavelength giving greater sensitivity to compounds with non-conjugated chromophores.

The TLC tests for tedanolide and the bryostatins were not repeated with the later fractions due to the lack of material.

The antiviral fractions from the chromatography of T14.23 and T14.34 were diluted to 20ml and submitted for P388 assay. On the assumption that the two fractions each contained 2µg of the active component, the IC<sub>50</sub> values were 28pg/ml and 40pg/ml respectively.

### ***Structure of the Antiviral Components of *Tedania connectens****

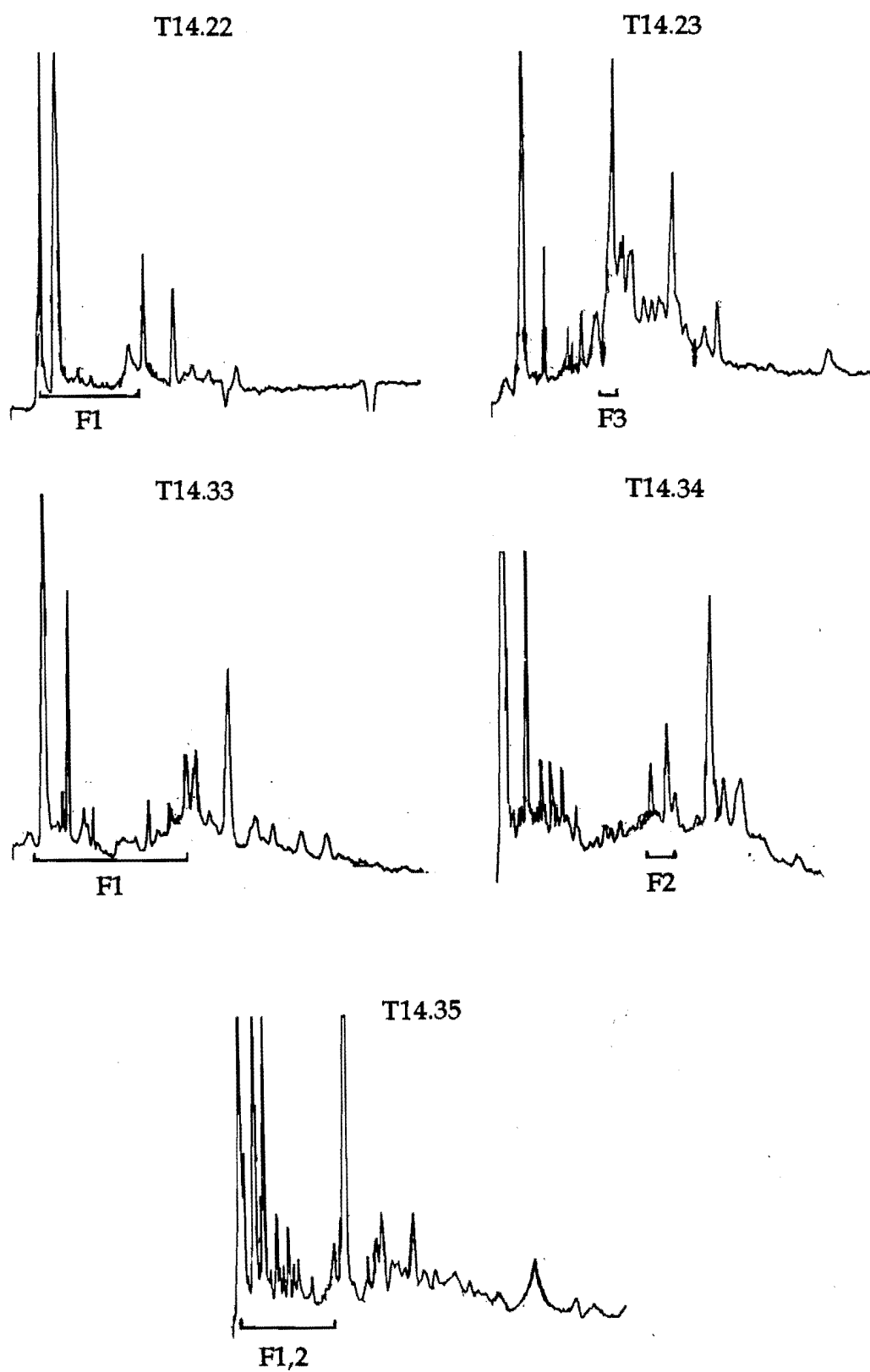
---

The identity of the antiviral compounds remained unclear until the penultimate step in the purification process. A number of possibilities had been considered at earlier stages, but in retrospect, the components in question were undoubtedly present at too low a level to be detected by NMR and mass spectrometry.

An analysis of the gel permeation results gave a value for the molecular mass, which was consistent with the results of the mass spectroscopy described below. The peak in the antiviral activity was estimated to be at a volume of 120ml. Using the elution volumes obtained for PEG 4000 and benzophenone as calibration points, a plot of elution volume against log of the mass gave a molecular weight of approximately 550 for the active compounds.

### HPLC Chromatograms of T14.22 - T14.35

---



The  $^1\text{H}$ -NMR spectra of samples T11, T12 and T14 were examined for the presence of a number of compounds, known to have biological activity. Although the spectra of these samples, run in  $\text{CD}_3\text{OD}$  and  $\text{CDCl}_3$ , showed a number of sharp peaks, the samples were clearly still complex mixtures.

The sponge had been identified as belonging to the *Tedania* genus by this stage, so tedanolide was an obvious possibility. However, neither the methoxyl resonance at  $\delta_{\text{H}}$  3.3ppm, nor the sharp methyl resonances in the region  $\delta_{\text{H}}$  1.1 to 1.7 ppm, which are characteristic of tedanolide, were observed. Another test, used on sample T12, for the presence of tedanolide was silica gel TLC followed by visualisation with vanillin/sulphuric acid. With this spray tedanolide gives a characteristic pale yellow spot. No yellow spots were observed at any  $R_f$ .

The sponge from which the antiviral and cytotoxic mycalamides had been isolated occurs in the area where the *T. connectens* was collected, and the possibility of contamination could not be ruled out. Neither of the methoxyl resonances, at  $\delta_{\text{H}}$  3.55 and 3.29 ppm, which are characteristic of mycalamide A were observed, and the mass spectra of the HPLC fractions from T14 contained no ions corresponding to these compounds.

Another potential source of contamination were bryozoa of the *Bugula* genus from which the intensely cytotoxic series of compounds known as bryostatins have been isolated.<sup>74</sup> Bryozoa often grow in close association with sponges and the cytotoxic activity associated with a *Lissodendoryx* species has been shown to result from *Bugula neritina* growing in the sponge matrix.<sup>75</sup> *Bugula* species have been collected from the Otago Harbour, and bryostatins were therefore considered. No peaks were found in the above  $^1\text{H}$ -NMR spectra that indicated the presence of these compounds. The bryostatins are known to give a characteristic purple colour

---

74 Pettit, G.R., Herald, C.L., Doubek, D.L., Herald, D.L., Arnold, E. and Clardy, J., *J. Am. Chem. Soc.*, **104**, 6846 (1982).

75 Pettit, G.R., Kamano, Y., Herald, C.L., Schmidt, J.M. and Zubrod, C.G., *Pure & Appl. Chem.*, **58**, 415 (1986).

reaction when a silica gel TLC is visualised with an anisaldehyde spray. A TLC of T12 gave no spots of the correct colour or  $R_f$ .

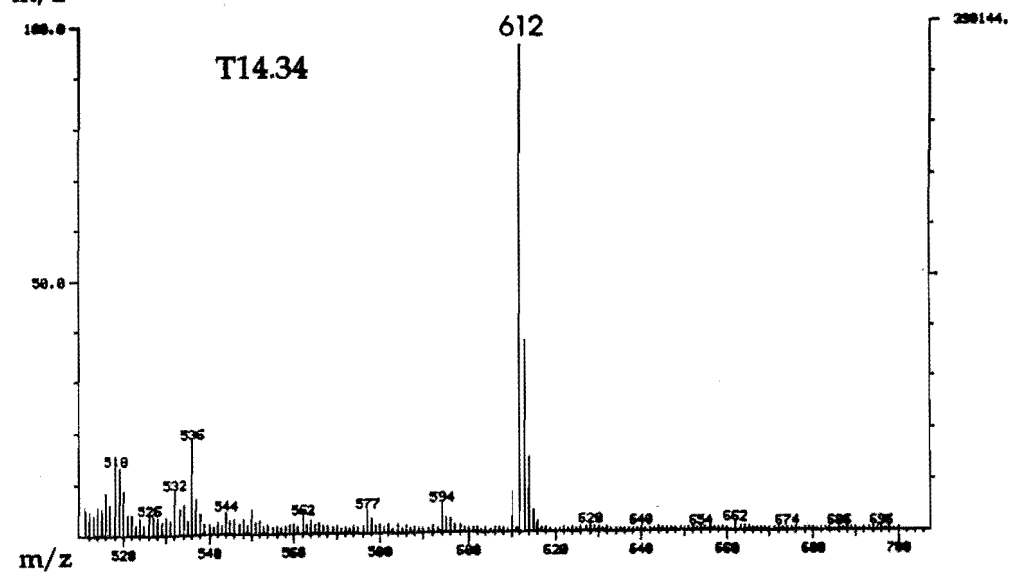
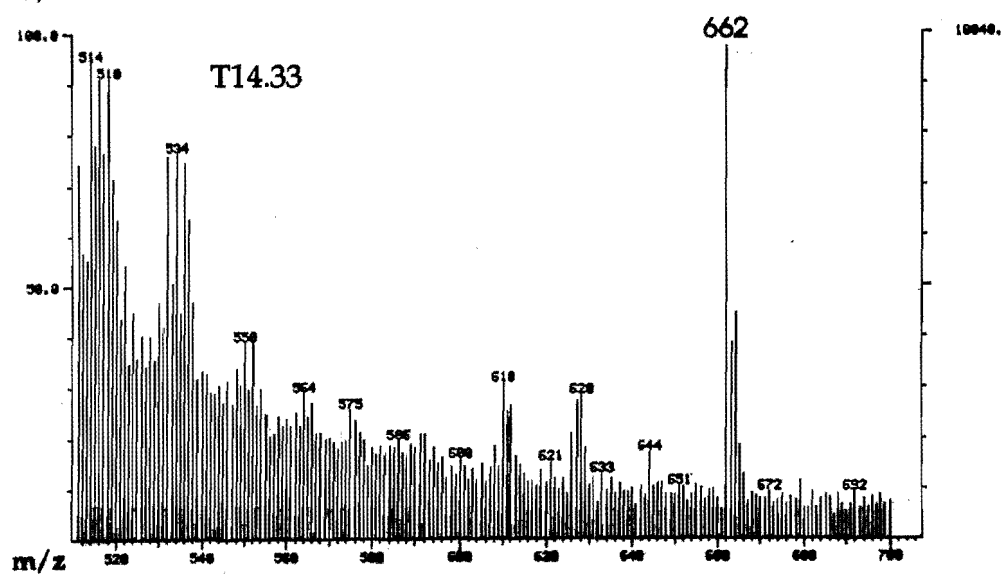
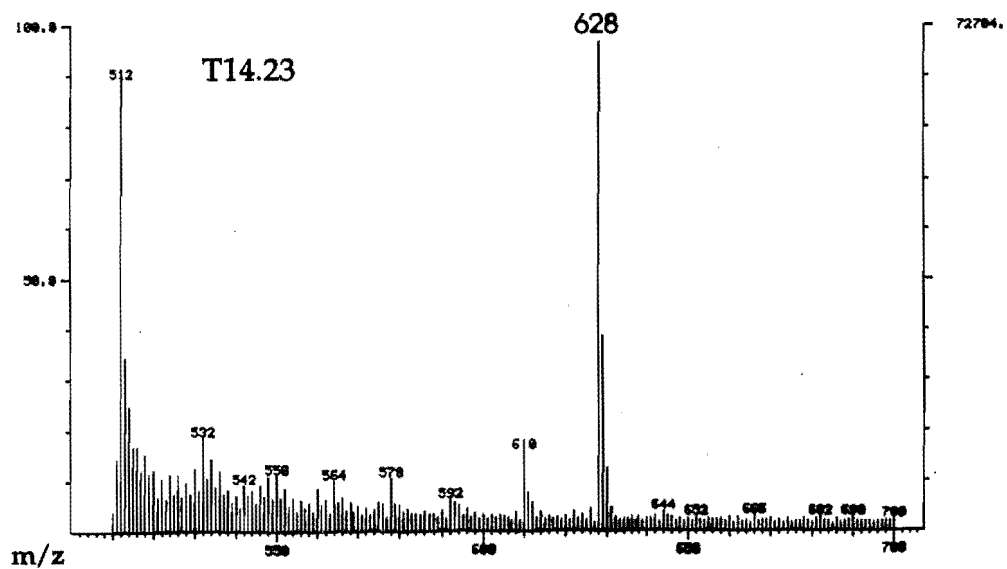
The conclusion that bryostatins or mycalamides were not the source of the antiviral activity was further supported by the observation that the extracts of new specimens of *T. connectens*, that were known to be free from contamination by other species, also exhibited strong antiviral activity.

Mass spectra were recorded for a number of the fractions derived from samples T12 and T13. DCI mass spectrometry with ammonia as the reagent gas was found to be the most successful of the DCI and DEI systems tried, although spectra were also obtained in negative ion mode with methane as the reagent gas. Under positive ion DCI/ammonia conditions  $M^+$ ,  $M+1^+$  and  $M+NH_4^+$  ions may be observed. In negative ion spectroscopy electron capture to give  $M^-$  is the most common process. The high mass region of positive ion DCI/ammonia spectra for samples T14.23, T14.33 and T14.34 are shown overleaf.

The first results obtained were for fraction eight of the silica gel column of T12. The negative ion DCI/methane spectrum showed strong high mass ions at  $m/z$  594, 610 and 644. Two main ions were observed in positive ion DCI/ammonia spectrum, at  $m/z$  612 and  $m/z$  628, with a much smaller ion at  $m/z$  662. The three positive ion peaks were respectively 18 AMU greater than those observed in the negative ion spectra, which suggested they were  $M+NH_4^+$  ions.

The number of exchangeable hydrogen atoms in a molecule or a fragment may be determined by using deuterated ammonia as the DCI reagent gas. When compared with the positive ion ammonia spectrum the mass of the  $M+ND_4^+$  ion is of course four mass units greater than the corresponding  $M+NH_4^+$  ion, but in the conditions of the mass spectrometer source each exchangeable hydrogen atom will be replaced by a deuterium atom. Hence the mass of the ion will be also increased by one mass unit for each such hydrogen.

The presence of the  $m/z$  610 (and 628) ion suggested the possibility that tedanolide was present in the sample. The observation of an ion at  $m/z$  636 in the positive ion

**DCI/Ammonia Mass Spectra for T14.23, T14.33 and T14.34**



DCI/ND<sub>3</sub> indicated that the compound contained four exchangeable protons, which is also consistent with the hypothesis that tedanolide, which has four hydroxyl groups, was present. The peak at  $m/z$  594 (and 612) may have been due to another compound, although the loss of oxygen by epoxides is not unknown.<sup>76</sup> A peak at  $m/z$  619 suggested that this compound had three exchangeable hydrogen atoms. No evidence for the presence of mycalamides, at  $m/z$  503 ( $M+NH_4^+$  521) and 517 ( $M+NH_4^+$  535) for A and B respectively, was observed in any of the spectra.

DCI/ammonia positive ion spectra were then obtained for T14.19, T14.20, T14.22 to T14.24 and T14.33 to T14.35. The results indicated that the ions observed in the previous set of spectra were attributable to three different compounds. No high mass ions were recorded for fractions T14.19 and T14.22. The spectrum T14.20 contained the previously observed ions at  $m/z$  612 and 628. The spectrum of T14.23 had an intense  $m/z$  628 peak and a weaker  $m/z$  610 peak. Strong ions at  $m/z$  662 and 612 were observed in the spectra of T14.33 and T14.34 respectively. A weaker ion at  $m/z$  586 was observed in the spectrum of T14.35, but examination of the total ion current plot for this fraction, and HPLC analysis suggested it was probably a complex mixture in which the antiviral components were present at a lower level than in the other samples.

The intensities of the  $m/z$  662 and 664 ions in the spectrum of T14.33 suggested the presence of a mono-chlorinated compound. The ion at 662 was consistent with the hypothesis that this compound was related to the compound with a mass of 610 by the substitution of a hydrogen atom with a chlorine atom. The isotopic pattern for chloro-tedanolide was calculated<sup>77</sup> and a close match of the intensities of the peaks at  $m/z$  662, 663, 664 and 665 was obtained.

It was clear that high-resolution mass spectra would be required to resolve the question of the identity of these compounds. High-resolution positive ion FAB mass spectra were measured for samples T14.23, T14.33 and T14.34, using xenon

---

<sup>76</sup> Pannell, L.K., personal communication.

<sup>77</sup> Using the program MassCalc, which is described later in this thesis.

and a thioglycerol matrix. No  $m/z$  610 ion was obtained for T14.23. The ion at  $m/z$  667.2909 in the spectrum of T14.33 was consistent with the molecular formula of a chlorinated tedanolide. On the assumption the compound in sample T14.34 is a deoxy derivative of tedanolide, ions corresponding to  $M-OH^+$  ( $m/z$  577.3328) and  $M+Na^+$  ( $m/z$  617.3311) were observed.

T14.22, T14.23 and T14.33 to T14.35 were each chromatographed once more, to obtain what was hoped would be nearly pure samples of the compounds. It was intended to obtain high-resolution mass measurements and tandem mass (MS/MS) spectra of the ions in these samples. Unfortunately, at the time of writing, a facility capable of making the required high-resolution mass measurements was not available.

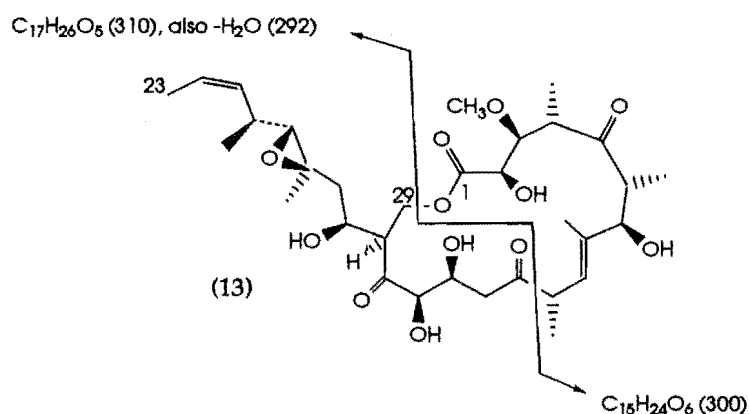
The MS/MS technique, which employs two mass spectrometers connected in series, allows the mass spectrum of each ion in a mass spectrum to be determined. Not only does it provide solid evidence for fragmentation pathways, but also MS/MS permits reliable information to be obtained from impure samples. It was hoped that MS/MS spectroscopy would provide sufficient information to confirm the structural assignments. Comparison of the MS/MS spectra of tedanolide and the two supposed tedanolide analogues would probably permit the sites of the changes in the substitution to be determined.

An EIMS spectrum of tedanolide, isolated from *T. ignis*, was obtained,<sup>78</sup> and it is hoped that this information will provide a basis for interpreting MS/MS data when it becomes available. Some fragments were tentatively identified and are shown overleaf.

Although the mass spectrometry results described above do not represent conclusive proof of the presence of tedanolide or an isomer, a deoxytedanolide and a mono-chlorotedanolide, these and earlier data are consistent with the proposed identification.

---

<sup>78</sup> Schmitz, F.J., personal communication.



The presence and absence of antiviral activity in samples, following the methylation and acetylation experiments, may also be rationalised in terms of the structure of tedanolide. Diazomethane would not be expected to react with tedanolide, and no change in the activity was noted. On the other hand the four secondary alcohol groups found in the tedanolide molecule would be susceptible to acetylation under the conditions used, which is consistent with observed loss of activity. Base hydrolysis was used on each of the above sets of reaction products in an attempt to regenerate the original compounds. The hydrolysis failed to restore any activity to the acetylation products and completely eliminated the antiviral activity of the methylation products. Under the conditions used tedanolide would be vulnerable to hydrolysis of the ester at C1, and possibly the epoxide at C29.

The DCI/ND<sub>3</sub> spectra gave the numbers of exchangeable hydrogen atoms for the compounds with masses of 610 and 594, four and three respectively. This is consistent with the assignment of these compounds as tedanolide and an analogue of tedanolide with only three hydroxyl groups.

Finally it seems unlikely that the species would contain an unrelated compound of which also has cytotoxic activity three orders of magnitude stronger than

compounds such as the mycalamides which are considered to be strongly active compounds.

### ***Concluding Remarks***

---

The purification of the active components from *T. connectens* exemplifies many of the difficulties encountered in bio-assay directed isolation work.

Species, or at least genus, identification should be one of the first criteria used in the selection of species to be studied. Without taxonomic information there is the risk both of repeating published work, and of extracting mixtures of organisms of similar appearance. In this instance it is fortunate that there were not species of similar appearance in the collection area. Correct identification of the sponge in this study, at the outset, would have probably eliminated it from consideration. In this case the amount of effort already committed to the isolation work when the identity of the sponge became known, was a major factor in the decision to continue the investigation.

Secondly, the intensity of the bio-activity of the active compounds presented several problems. The first was the scale of the extraction and initial chromatographic steps. Without the cyclone evaporator and the development of the reverse-phase partitioning techniques, dealing with the large quantity of material would have posed a much more difficult problem. Secondly, the concentrations of tedanolide, in a sample, required to produce a positive result in the assay meant that its spectroscopic characteristics were masked until almost the final steps in the purification. The unparalleled and unanticipated intensity of the biological activity of these compounds was very misleading. The large scale extraction which was expected to yield milligrams gave only micrograms of the active component. The purification involved eleven major steps. Assuming each procedure had an eighty percent yield, the overall yield was approximately ten percent, hence the level in the wet sponge would be about four parts per billion. In contrast Schmitz *et al*<sup>13</sup> reported tedanolide at a level of one part per million.

It was noted in the Introduction that the antiviral assay used in this study gave a positive result for a number of protein synthesis inhibitors, as well as for antiviral compounds. This may well be the reason for the activity of tedanolide in the system.

The origin and function of tedanolide and the related compounds is unclear. They may be metabolites of the sponge itself, or arise from symbiotic or commensal bacteria within the matrix of the sponge. The bacterial origin of some *Tedania* metabolites was noted in the introduction to this section. It is also possible that the active compounds are concentrated by the sponge from a dietary source. At one point in the isolation, contamination by other sponges such as *Mycale* was considered, however the activity of subsequent collections of known purity ruled this out, as did the absence of evidence for mycalamide. Since tedanolide occurs in Caribbean *T. ignis*, and since samples of *T. connectens* from the very different Kaikoura environment had similar levels and type of biological activity to the Otago samples, and the active extracts of the Kaikoura sponges displayed similar chromatographic behaviour it seems likely that these compounds are either sponge metabolites or metabolites of an organism that has a very specific association with the sponge. Over forty samples of sponge, spanning five *Tedania* species, have been collected at sites from the Auckland Islands to Leigh, north of Auckland. With the exception of *Tedania* n. sp. A, examples of all the species have exhibited antiviral activity. Considerable variation was observed in the activity shown in the screening assays. However, no correlation between the activity and either locality, habitat or season could be discerned. A link between the production of cytotoxic/antiviral compounds and environmental challenges, such as crowding by other species, or infection by micro-organisms, has been proposed,<sup>79</sup> but no data is available for this genus.

---

79 Battershill, C., *The Search for Antiviral Chemicals from New Zealand Marine Benthos - Ecological Clues* presented to the 1986 NZ Marine Sciences Society conference.

## Thyrsiferol

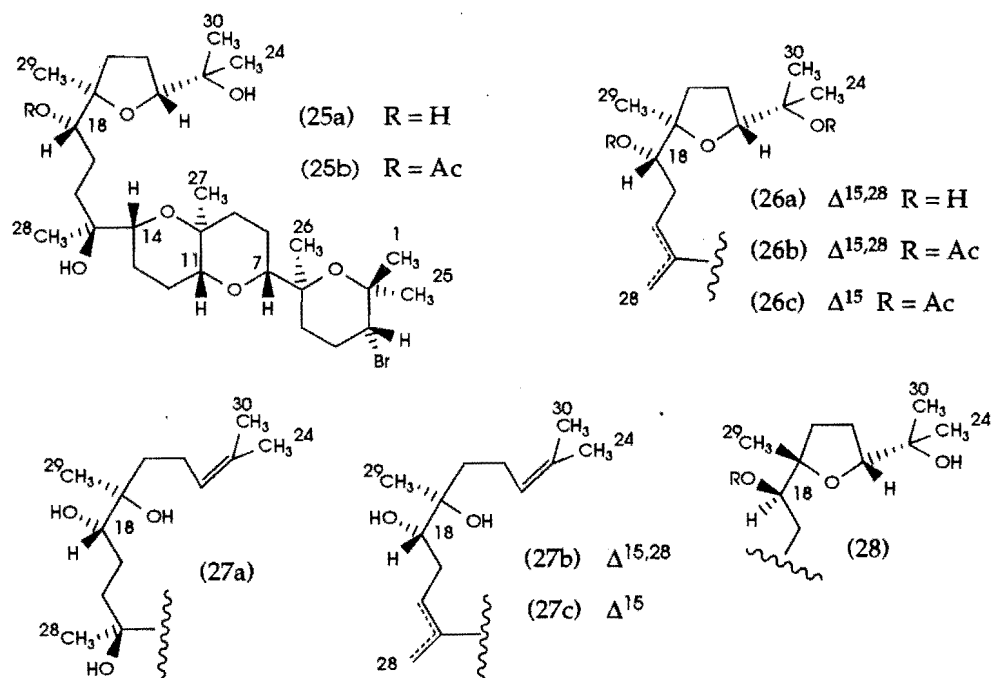
---

Red marine algae of the genus *Laurencia* have provided a extensive source of interesting halogenated metabolites. Several hundred compounds, including a number of new structural types, have been reported. The majority of these metabolites are sesquiterpenoids.<sup>80</sup>

Thyrsiferol (25a) is a squalene-derived polyether, the first of the few triterpenoids which have been isolated from this genus. Since its discovery in 1975<sup>81</sup> there has been much interest in this compound both for its antiviral and cytotoxic activity, and as a novel synthetic target.<sup>82,83,84,85</sup>

Several related compounds have also been isolated. 15(28)-Anhydrothyrsiferol (26a) was isolated from *L. pinnatifida* from the Canary Islands.<sup>86</sup> Thyrsiferyl 23-acetate was obtained from a methanol extract of *L. obtusa*,<sup>87</sup> and 15(28)-anhydrothyrsiferyl diacetate (26b), 15-anhydrothyrsiferyl diacetate (26c) and the magireol series (27a), (27b), (26c) were isolated in a subsequent study.<sup>88</sup>

- 
- 80 Erickson, K.L., *Constituents of Laurencia in Scheuer, P.J., Marine Natural Products: Chemical and Biological Perspectives*, Vol V, 132 (Academic Press : New York 1983).
- 81 Blunt, J.W., Hartshorn, M.P., McLennan, T.J., Munro, M.H.G., Robinson, W.T. and Yorke, S.C., *Tetrahedron Lett.*, 69 (1978).
- 82 Broka, C.A., Hu, L., Wen Jee Lee and Tong Shen, *Tetrahedron Lett.*, 28, 4993 (1987).
- 83 Hashimoto, M., Kan, T., Yanagiya, M., Shirahama, H. and Matsumoto, T., *Tetrahedron Lett.*, 28, 5665 (1987).
- 84 Hashimoto, M., Kan, T., Nozaki, K., Yanagiya, M., Shirahama, H. and Matsumoto, T., *Tetrahedron Lett.*, 29, 1143 (1988).
- 85 Broka, C.A. and Yi-Tsong Lin, *J. Org. Chem.*, 53, 5876 (1988).
- 86 Gonzalaz, A.G., Arteaga, J.M., Fernandez, J.J., Martin, J.D., Norte, M. and Ruano, J.Z., *Tetrahedron*, 40, 2751 (1984).
- 87 Suzuki, T., Suzuki, M., Furusaki, A., Matsumoto, T., Kato, A., Imanaka, Y. and Kurosawa, E., *Tetrahedron Lett.*, 26, 1329 (1985).
- 88 Suzuki, T., Takeda, S., Suzuki, M., Kurosawa, E., Kato, A. and Imanaka, Y., *Chem. Soc. Japan, Chem. Lett.*, 361 (1987).



Bio-assay directed analysis of an extract of *L. venusta*, from Okinawa, led to the isolation of the antiviral compound venustatriol (28) (activity against vesicular stomatitis and *Herpes simplex* type I viruses). This compound differs from thyrseferol only in the stereochemistry at C18 and C19. The structure, including the absolute configuration, was determined by single crystal X-ray crystallography.<sup>89</sup> The authors make the not unreasonable assertion that the absolute configuration of C3, C6, C7, C10, C11, C14 and C15 for thyrseferol is the same as that venustatriol, but no evidence was presented to support that claim. Blunt *et al* suggested the opposite absolute configuration, but their anomalous dispersion data was not conclusive. A total synthesis of venustatriol has been reported.<sup>90</sup>

Interest was aroused by the observation that the oxidation products of thyrseferol possessed intense cytotoxic activity.<sup>91</sup> In this study the complete assignment of the

89 Sakemi, S., Higa, T., Jefford, C.W. and Bernardinelli, G., *Tetrahedron. Lett.*, **27**, 4287 (1986).

90 Corey, E.J. and Ha, D., *Tetrahedron Lett.*, **29**, 3171 (1988).

91 Thomas, F.N., BSc Hons Project (University of Canterbury : 1986).

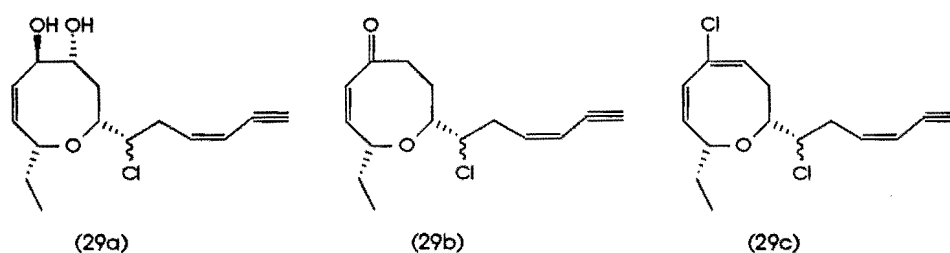
$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of thyrsiferyl 18-acetate (25b), and the partial assignment of thyrsiferol, was undertaken, as an adjunct to chemical modification work.

### Isolation of Thyrsiferol

*L. thyrsifera* grows abundantly on the intertidal margin at Seal Reef, Kaikoura. The thyrsiferol used for the NMR and chemical modifications studies, described in this section, was obtained by a combination of reverse-phase and silica gel chromatography of a methanol/dichloromethane extract of the alga. Final purification was by recrystallisation from ethyl acetate/pet. ether.

NMR spectroscopy indicated the presence of lauroxane enynes, (for example 29a, 29b, 29c) of the type previously reported from this species,<sup>92</sup> in some semi-crystalline fractions. Extensive attempts were made to isolate these compounds, but pure compounds could not be obtained due to the limited material available.

Thyrsiferol and the enyne related compounds are awkward to deal with by HPLC. The most satisfactory method is silica gel chromatography. A methanol/chloroform solvent system gives the best resolution, but is not satisfactory for either refractive index or UV detectors. A reverse-phase chromatography method using a



cyanopropyl column, eluted with 50% acetonitrile/water was developed. This system gave satisfactory results for sub-milligram separations, but was limited in preparative work by the relative insolubility of these compounds in the solvents.

92 Lake, R.J., PhD Thesis (University of Canterbury : 1982).



### **Oxidation of Thysiferol**

Attempts were made to reproduce the results of Thomas, with the aim of isolating the strongly cytotoxic compounds obtained from the oxidation of thysiferol. It was initially supposed that the thysifer-18-one was responsible for the biological activity. Repeated attempts, using several oxidation methods, failed to give the increase in cytotoxicity reported by Thomas, and none of the methods gave a smooth conversion of thysiferol to thysifer-18-one.

Oxidation of thysiferol using freshly prepared Jones' reagent<sup>93</sup> resulted in a mixture of at least seven compounds that exhibited little antiviral activity and no cytotoxicity, in the antiviral assay. A similar, although slightly less complex mixture was obtained from the treatment of an acetone solution of thysiferol with two drops of perchloric acid. The instability of thysiferol under these conditions is almost certainly due to the number of tertiary hydroxyl groups in the molecule, which offer the opportunity for dehydration or re-arrangements.

The milder conditions obtained using pyridinium chlorochromate, buffered with sodium acetate produced a much simpler mixture than the previous attempt. Although the IR spectrum contained evidence for the formation of a ketone, no enhancement of the cytotoxicity was obtained.

Oxidation of thysiferol using dimethylsulphoxide (DMSO) as the oxidising reagent was also attempted. The method of Moffat<sup>94</sup> is considered to involve the nucleophilic attack of DMSO on protonated dicyclohexyldicarbodiimide to give an ionic intermediate, which is in turn attacked by the substrate alcohol, ultimately forming dicyclohexylurea, dimethylsulphide and the ketone. A similar method, involving the formation of an intermediate from DMSO and oxalyl chloride at low

---

93 Fieser, L.F. and Fieser, M., *Reagents for Organic Synthesis*, 142 (John Wiley & Sons : New York 1967).

94 Pfizner, K.E. and Moffat, J.G., *J. Am. Chem. Soc.*, **87**, 5670 (1965).

temperature, was reported by Swern.<sup>95</sup> Both methods were attempted, but no reaction was obtained.

Unfortunately none of the reaction mixtures exhibited any cytotoxicity that could not be explained in terms of the residual thyriferol in the sample.

### Epimerisation of Thyriferol

Attempts were made to prepare 18-epi-thyriferol. It was hoped that comparison of the cytotoxic activity of thyriferol, 18-epi-thyriferol and venustatriol (28), would give an indication of the relationship of this region of the molecule to the biological activity.

In the first of two approaches tried, the 18-mesylate was formed, in the hope that it would be possible to displace this group under  $S_N2$  conditions. The reaction to form the mesylate proceeded smoothly to give the derivative in high yield. However, none of the attempts to displace the group succeeded, with either no reaction taking place, or with the decomposition of the mesylate into a number of products.

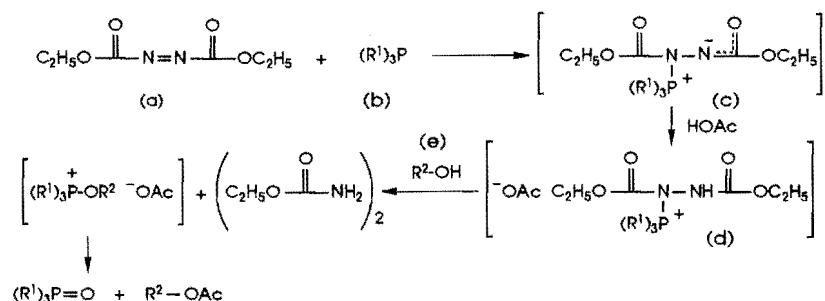
The second proposed strategy was to use the stereospecific reaction of Mitsunobu<sup>96</sup> to form the 18-epi acetate, and then to hydrolyse this ester. This reaction is believed to involve the addition of triphenyl phosphine (b) to diethyl azodicarboxylate (a) to give a quaternary phosphonium salt (c). Protonation of the salt with acetic acid is followed by the formation of an alkoxy phosphonium salt (d) with the substrate (e). Finally  $S_N2$  displacement gives the ester with an inversion of the stereochemistry. No reaction was obtained, in several trials, which was undoubtedly due to steric factors in this hindered region of the molecule<sup>97</sup>

---

95 Mancuso, A.J., Shui-Lung Huang and Swern, D., *J. Org. Chem.*, **43**, 2480 (1978).

96 Mitsunobu, O., *Synthesis*, **1** (January 1981).

97 Bose, A.K., Bansilal, Hoffman, W.A. III and Manhas, M.S., *Tetrahedron Lett.*, **18**, 1619 (1973).

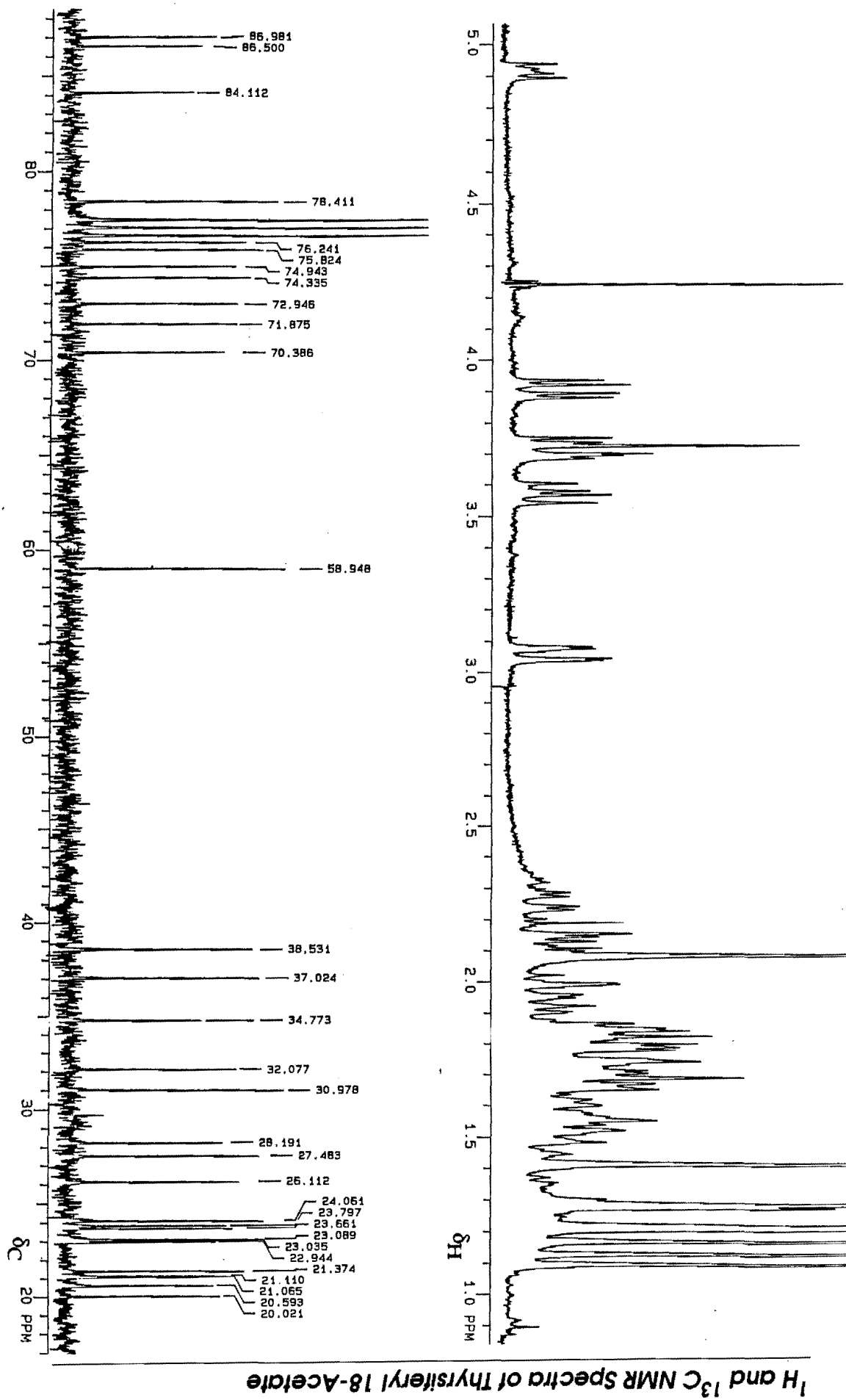


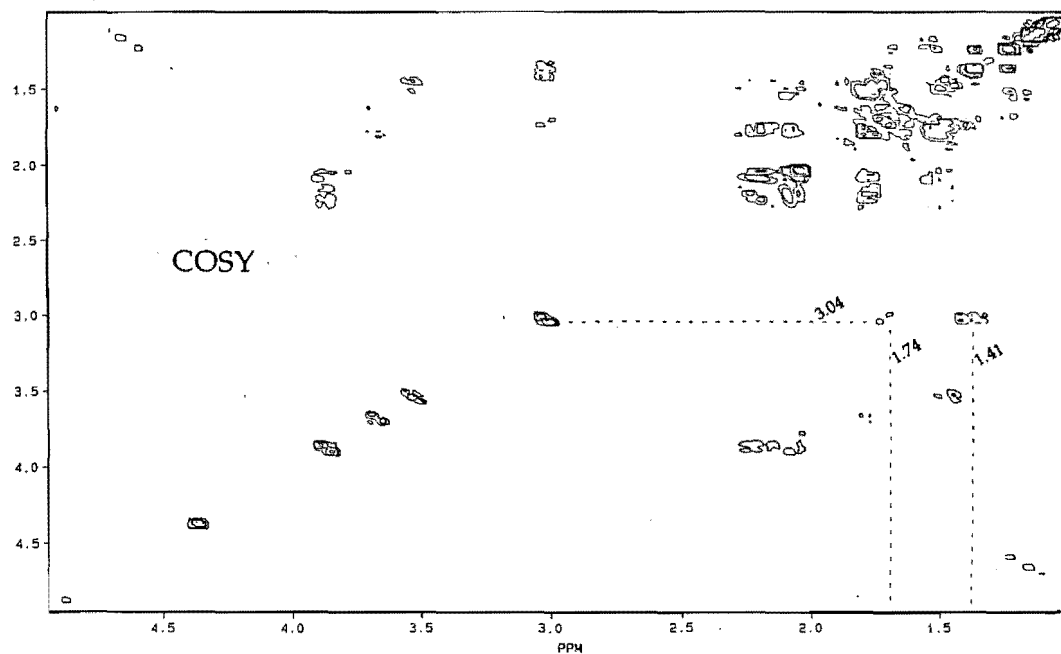
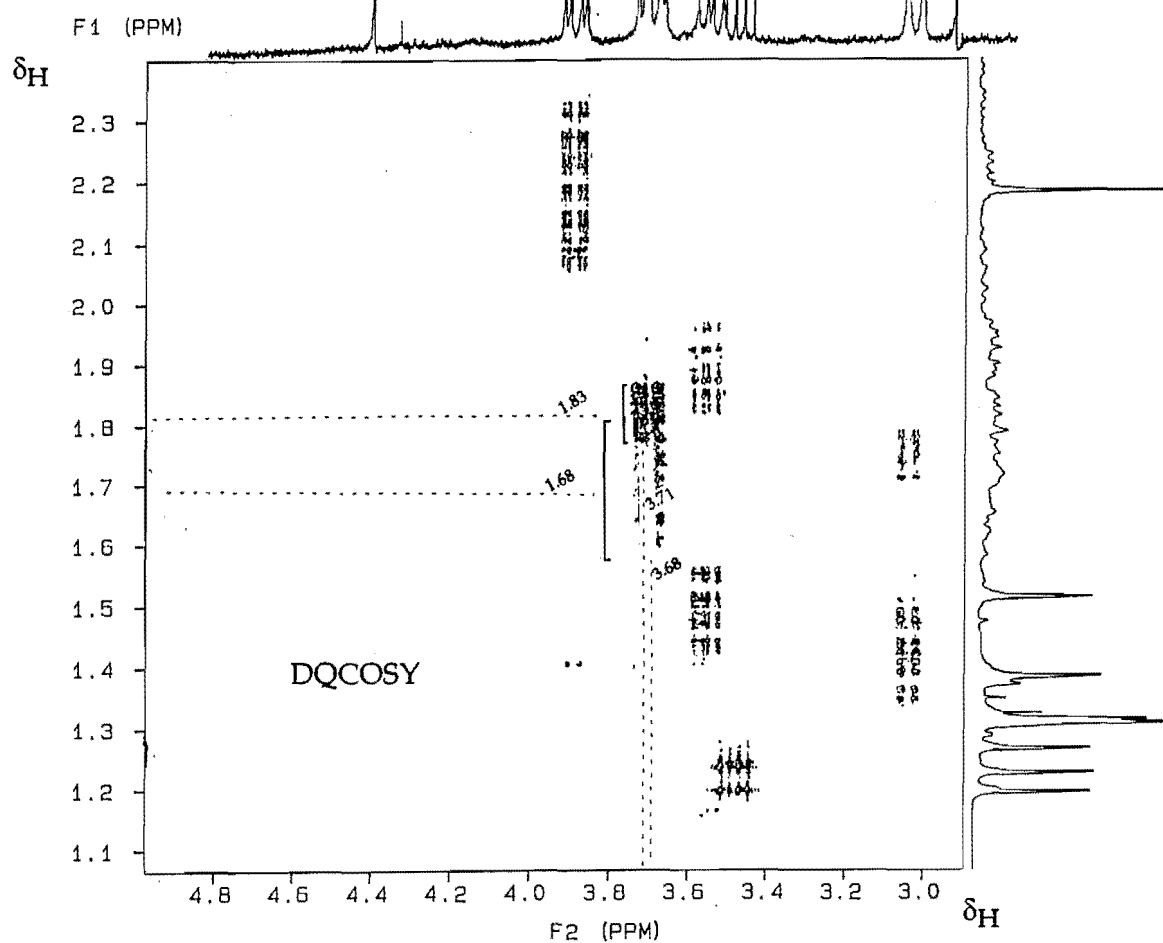
Had a satisfactory method for synthesising thyrifer-18-one been devised, it may have been possible to form 18-epi-thyriferol by reducing the ketone, then separating the epimers by chromatography. Exhaustion of the limited supply of thyriferol precluded further chemical studies.

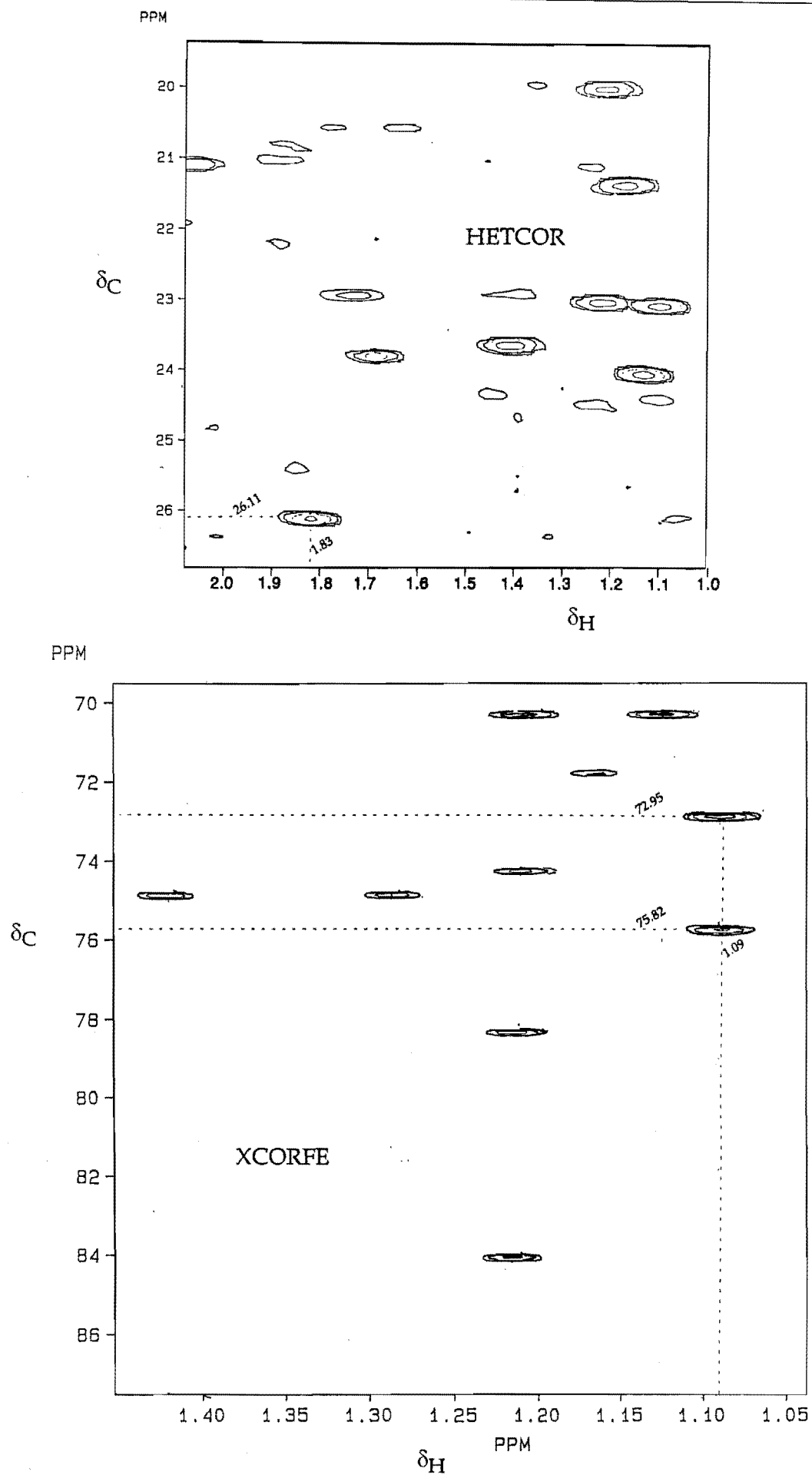
### <sup>13</sup>C and <sup>1</sup>H-NMR Spectra of Thyriferyl 18-Acetate

The <sup>13</sup>C-NMR and <sup>1</sup>H-NMR spectra of thyriferyl 18-acetate were completely assigned using 2D and difference nOe techniques. Selected portions of these spectra are shown on the succeeding pages. The 2D spectra are shown as contour plots, with the proton spectrum on both axes for the COSY and DQ-COSY spectra, and the proton spectrum plotted against the carbon spectrum for the HETCOR and XCORFE spectra.

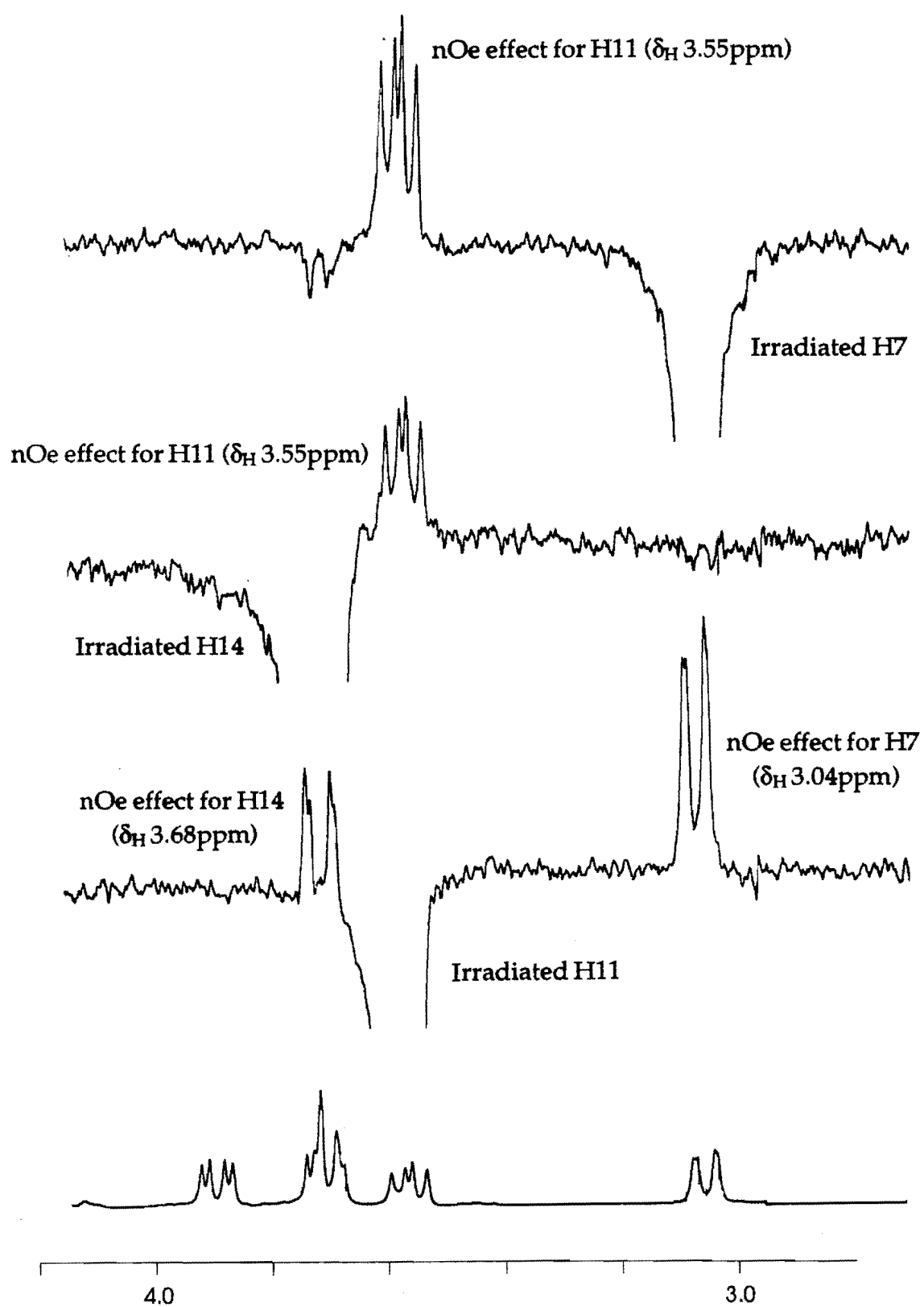
The COSY and DQ-COSY spectra show the spin-spin couplings between hydrogen atoms in the molecule, and hence, which hydrogens are bonded to adjacent carbon atoms. The portion of the spectrum, on page 70, shows how the connectivity from H7 to H8r and H8s was derived. The long range coupling between the *gem* dimethyl groups attached to C2 and C23 may be clearly seen in the upper right corner of the spectrum. The double-quantum filtered spectrum (DQ-COSY) gives much sharper peaks, and was used to obtain the connectivities for H14 and H22 which could not be derived from the COSY spectrum. The carbon atom to which a hydrogen atom is bonded, and *vice versa*, may be identified from the HETCOR spectrum. Examination of the homonuclear and heteronuclear spectra, therefore, allowed the assignment of the CH<sub>2</sub>CH<sub>2</sub> chains, and the assignment of the hydrogen atoms to their respective carbon atoms. In the spectra shown, the



**COSY and DQCOSY Spectra of Thyrsiferol 18-Acetate** $\delta_H$  $\delta_H$ 

**HETCOR and XCORFE Spectra of Thyrsiferol 18-Acetate**

### Selected nOe Difference Spectra of Thyrsiferol 18-Acetate



correlations for H14 and H22 are marked, by way of illustration of the techniques involved.

The XCORFE<sup>98</sup> spectrum is similar to the HETCOR spectrum, but shows the coupling between hydrogen atoms and carbon atoms, one or more bonds away. The data allowed the assignment across fully substituted centres such as C15, where, of course, the connectivity cannot be derived from COSY data. The XCORFE spectrum also gave further support for some of the connectivities already inferred from COSY and HETCOR data. The XCORFE correlations between C14 ( $\delta_C$  75.82) and C15 ( $\delta_C$  72.95) to H28 ( $\delta_H$  1.09) are marked on the spectrum shown on page 71, as illustrations of how the information may be extracted

Finally, difference nOe spectroscopy was used to give information about the spatial relationship between hydrogen atoms in the molecule, by showing the direct dipole-dipole interactions between atoms which were close to one another. This data enabled the assignment of every *pro-r* and *pro-s*  $^1H$  resonance. In the example shown (see p72), the positive nOe effect for H11, created by irradiation of H7 and H14 is highlighted.

The degree of substitution of the carbon atoms was determined using the DEPT experiment, which allows subspectra to be plotted for CH, CH<sub>2</sub> and CH<sub>3</sub> groups.

Although the starting points for the assignment were based on the unique chemical shifts of two resonances, the complete assignment stands on its own without any recourse to chemical shift analogies. The assignment of thyrsiferyl 18-acetate was made more complicated by the presence of five CH<sub>2</sub>CH<sub>2</sub> units which contributed to the complex envelope from  $\delta_H$  1.0 to 2.3 ppm.<sup>99</sup>

---

98 Reynolds, W.F., Hughes, D.W., Perpich-Dumont, M. and Enriquez, R.G., *J. Magn. Reson.*, **64**, 303 (1985).

99 Blunt, J.W., McCombs, J.D., Munro, M.H.G. and Thomas, F.N., *Magn. Reson. in Chem.*, (in press)



The connectivities implied by the spectroscopic data used for this assignment are summarised in the correlation table following the table of chemical shift assignments, overleaf. This style of diagram was developed as a method of concisely presenting the spectroscopic data in a form suitable for publication. The subsequent diagram is a graphical representation of the information in the table. The colour of the arrows denotes the type of spectrum from which the connectivity was derived. With the exception of the nOe correlations, the direction of the arrows shows the way the assignment was built up. For example the assignments of C3 and H4 were made from the starting point of H3. For the nOe data the arrow head designates the hydrogen atom for which the enhancement was observed. A double headed arrow indicates that the nOe was observed in both directions.

The final diagram is a perspective drawing of the crystal structure, split in two for clarity, of thyrsiferol 18-acetate. These illustrations also appear in the fold-out section at the back of this thesis.

The assignment was made in the following manner. The resonance at  $\delta_C$  58.95 ppm, which is typical for a bromo-methylene group,<sup>100</sup> and was the only signal in this part of the  $^{13}C$ -NMR spectrum, was used as the starting point for the assignment. The HETCOR spectrum indicated a single attached proton resonance at  $\delta_H$  3.89 ppm. COSY data showed a correlation of this signal with resonances at  $\delta_H$  2.1 and 2.25 ppm, which were assumed to be the *geminal* protons at C4. This was supported by an XCORFE correlation between C3 and the proton at  $\delta_H$  2.1 ppm, and a HETCOR correlation of the two proton resonances with a single carbon signal ( $\delta_C$  28.19). The assignment of the signals for C5 was based on COSY correlations between the protons at  $\delta_H$  2.1 and 2.25 ppm and resonances at 1.53 and 1.84 ppm. An XCORFE correlation between C4 and the proton at  $\delta_H$  1.84 ppm supported this assignment. C5 was then assigned as  $\delta_C$  37.07 ppm from the HETCOR spectrum.

---

100 C6 of 6 $\beta$ -bromo-5 $\alpha$ -cholestane-3 $\beta$ ,5-diol 3-acetate was used as the model. Blunt, J.W. and Stothers, J.B., *Org. Magn. Reson.*, **9**, 439 (1977).

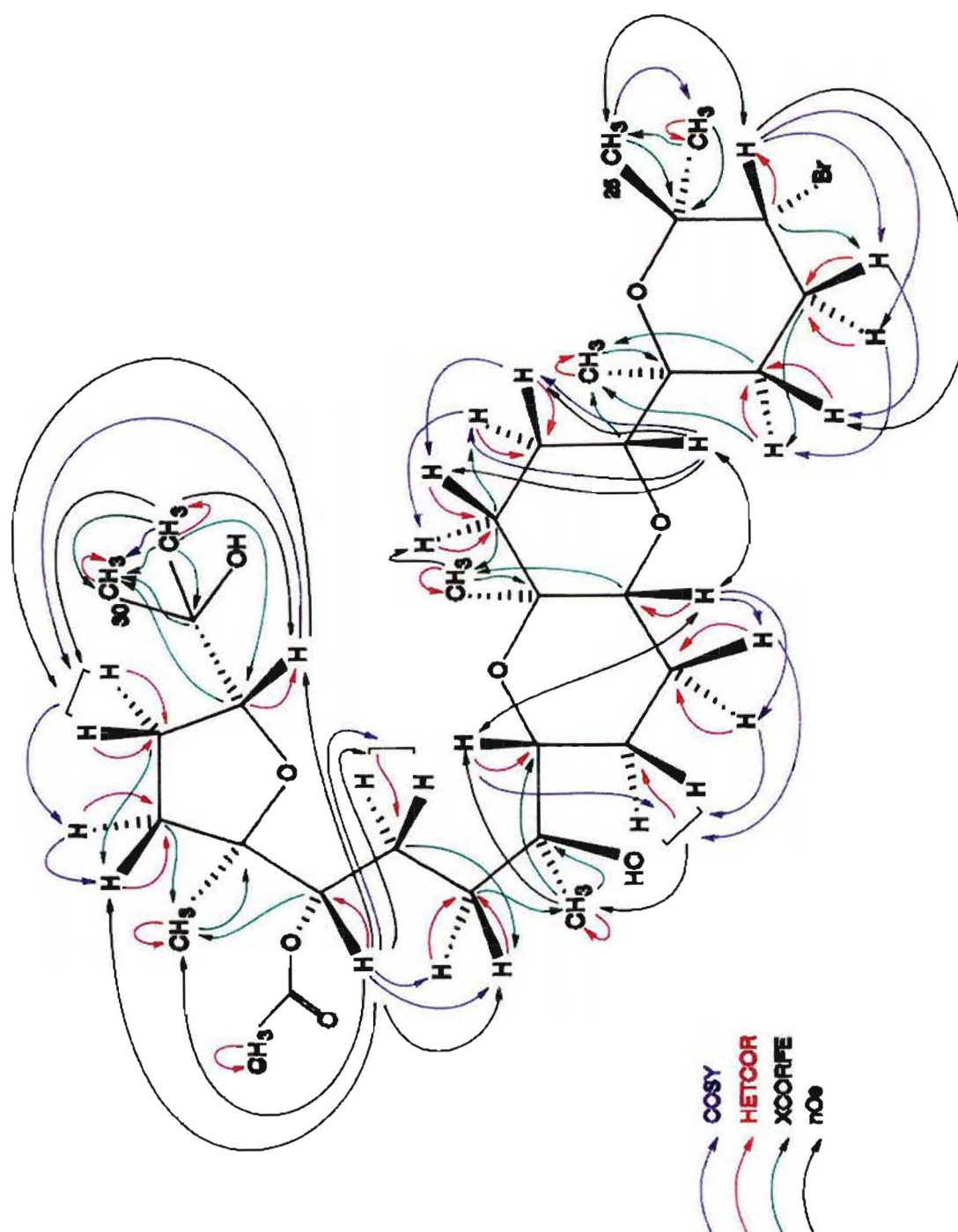
### Chemical Shift Assignments for Thyrsiferyl Acetate and Thyrsiferol

Thyrsiferyl 18-Acetate				Thyrsiferol			
	$\delta_c$	DEPT <sup>a</sup>	$\delta_H$		$\delta_c$	DEPT <sup>a</sup>	$\delta_H$
1	23.66	3	1.42		23.65	3	1.40
2	74.94	0	-		74.93	0	-
3	58.95	1	3.89, dd, $J = 12.4, 4.2$ Hz		58.96	1	3.89, dd, $J = 12.2, 4.3$ Hz
4	28.19	2	2.25r, 2.10s		28.20	2	2.24, 2.07
5	37.07	2	1.53r, 1.84s		37.02	2	1.79, 1.52
6	74.34	0	-		74.33	0	-
7	86.50	1	3.04, dd, $J = 11.0, 1.8$ Hz		86.50	1	3.04, dd, $J = 9.6, 2.3$ Hz
8	22.94	2	1.74r, 1.41s		22.96	2	?
9	38.53	2	1.78r, 1.56s		38.49	2	1.78, 1.53
10	71.88	0	-		71.92	0	-
11	76.24	1	3.55, dd, $J = 10.5, 6.6$ Hz		76.26	1	3.56, dd, $J = 11.3, 7.0$ Hz
12	21.07	2	1.48r, 1.91s		21.12	2	?
13	23.80	2	1.68r, 1.68s		25.43	2	1.58, 1.58
14	75.82	1	3.68, dd, $J = 11.1, 3.0$ Hz		76.05	1	3.73, dd, $J = 12.3, 3.1$ Hz
15	72.95	0	-		73.21	0	-
16	32.08	2	1.57r, 1.25s		32.31	2	2.1, ?
17	20.59	2	1.68r, 1.68s		20.68	2	?
18	78.41	1	4.90, dd, $J = 8.4, 5.2$ Hz		77.60	1	3.45, dd, $J = 9.8, 2.2$ Hz
19	84.11	0	-		86.00	0	-
20	34.77	2	1.68r, 1.93s		33.51	2	?
21	26.11	2	1.83r, 1.83s		26.57	2	1.85, 1.85
22	86.98	1	3.71, dd, $J = 8.1, 7.5$ Hz		87.40	1	3.76, dd, $J = 9.3, 6.5$ Hz
23	70.39	0	-		70.49	0	-
24	24.06	3	1.13		23.90	3	1.12
25	30.98	3	1.27		30.97	3	1.27
26	21.37	3	1.17		21.42	3	1.18
27	20.02	3	1.19		20.02	3	1.20
28	23.09	3	1.09		23.39	3	1.15
29	23.03	3	1.20		22.85	3	1.09
30	27.48	3	1.19		27.63	3	1.21
31	21.11	3	2.08		-	-	-

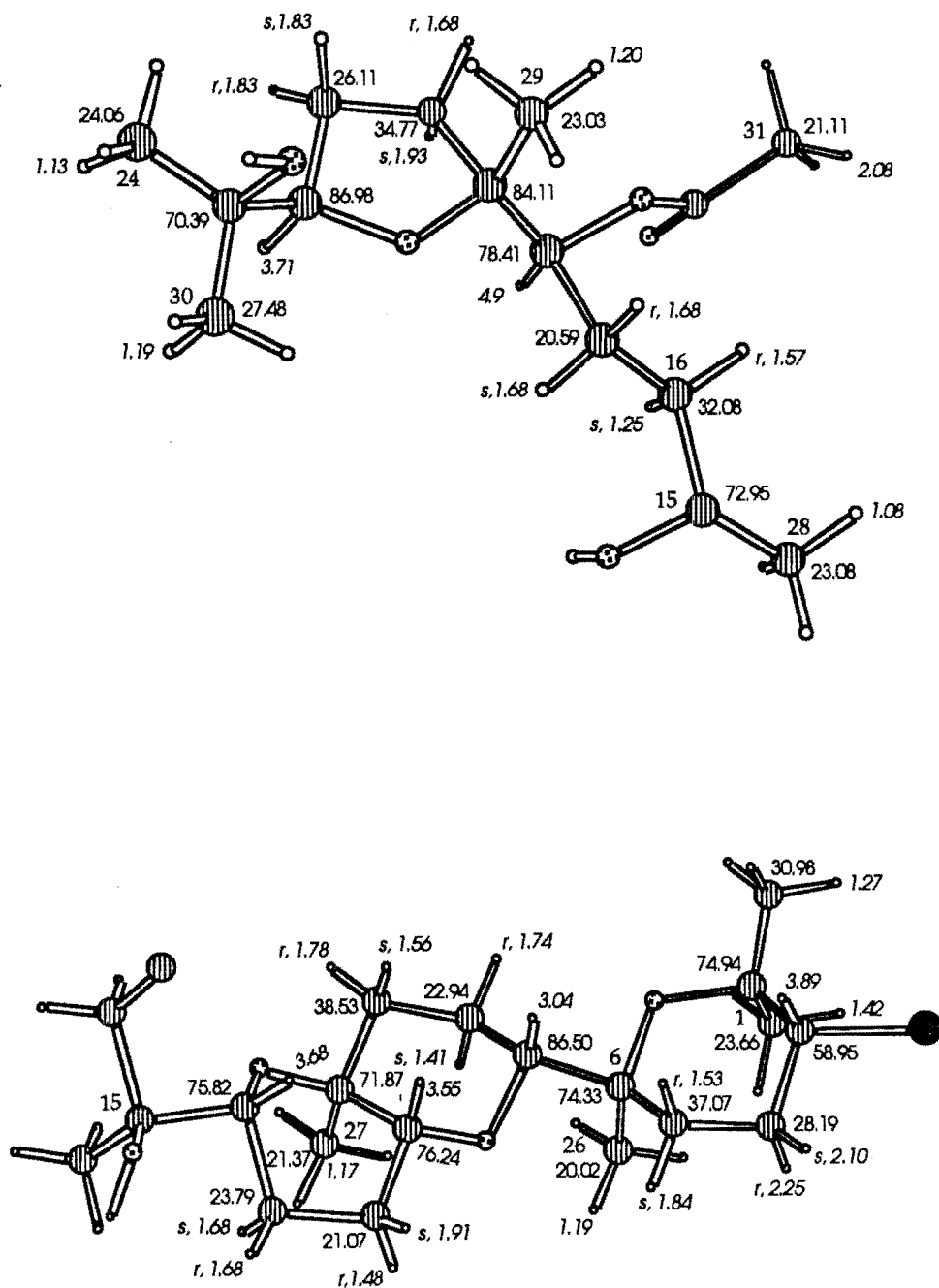
<sup>a</sup>Numbers of attached hydrogen atoms, determined by a DEPT experiment

[illegible]

The path by which the assignments were made is shown by tracing from previously assigned atoms, in the "Assignments From" column, to atoms in the "Assignments To" row. The type of correlation is indicated by the following symbols: C = COSY, ▲ = nOe, H = HETCOR, X = XCORFE, ■ = COSY + nOe

NMR Correlation Diagram for Thyrsiferol 18-Acetate

### Crystal Structure of Thyrsiferyl 18-Acetate



The assignment of the stereochemistry of the C3 C4 C5 fragment was based on nOe difference spectra. The *pro-s* (equatorial) proton at C4 and the *pro-r* (axial) proton at C5 are the closest to the axial H3 proton. Irradiation at  $\delta_{\text{H}}$  3.89 ppm produced enhancements of the signals at  $\delta_{\text{H}}$  2.1 and 1.53 ppm, but not those at 2.25 and 1.84 ppm, which allowed the assignment of H4r as 2.25 ppm, H4s as 2.1 ppm, H5r as 1.53 ppm and H5s as 1.84 ppm.

Assignment of the signals for the C26 methyl group was made possible by the observation of correlations in the XCORFE spectrum between C5 and  $\delta_{\text{H}}$  1.19 and between H5s and  $\delta_{\text{C}}$  20.02 ppm. This assignment was consistent with a HETCOR correlation found between  $\delta_{\text{C}}$  20.02 and  $\delta_{\text{H}}$  1.19 ppm, and further confirmed, once the central section had been assigned, by the XCORFE correlation with C7.

Thyrsiferyl acetate contains two *gem* dimethyl systems, one at C2 and the other at C23. They were readily identified from the long range couplings observed in the DQCOSY spectrum. The immediate environment of each set of *gem* dimethyl systems was elucidated as follows. In each case the carbon atoms were identified from the HETCOR spectrum. The XCORFE spectrum was then used to correlate the methyl proton resonances with that of the other methyl carbon atom in the geminal pair, and that of the carbon atom to which they were attached. One pair of methyls had  $^1\text{H}$ -NMR resonances at  $\delta_{\text{H}}$  1.42 and 1.27 ppm. Examination of the HETCOR spectrum showed correlations between  $\delta_{\text{H}}$  1.42 and  $\delta_{\text{C}}$  23.66 and between  $\delta_{\text{H}}$  1.27 and  $\delta_{\text{C}}$  30.98 ppm. Both sets of methyl protons showed XCORFE correlations with the carbon resonance of the other methyl group, and with the carbon resonance at  $\delta_{\text{C}}$  74.94 ppm.

The other *gem* dimethyl system had  $^1\text{H}$ -NMR signals at  $\delta_{\text{H}}$  1.13 and 1.19 ppm and HETCOR correlations were observed to carbon resonances at  $\delta_{\text{C}}$  24.06 and 27.48 ppm respectively. XCORFE correlations of the proton and carbon signals at  $\delta_{\text{H}}$  1.13 and  $\delta_{\text{C}}$  24.06 ppm with resonances of the other methyl group were found. Correlations between the methyl protons at  $\delta_{\text{H}}$  1.13 and the carbon resonance at  $\delta_{\text{C}}$  70.39 ppm, and between this carbon and the other methyl protons, at  $\delta_{\text{H}}$  1.19, were also observed.

One of the *gem* dimethyl systems was located by nOe difference experiments. Irradiation of the H3 proton enhanced the methyl signal at  $\delta_{\text{H}}$  1.27 ppm, but not that at 1.42 ppm. Conversely irradiation at 1.27 ppm enhanced the H3 signal. This result identified one *gem* dimethyl moiety as C1/C25, and allowed the assignment of the resonance at  $\delta_{\text{C}}$  74.94 ppm as being that of C2. Since the *pro-s* (equatorial) methyl group was much closer to H3 than the *pro-r* (2.55 Å vs 3.32 Å between the carbon atoms) the methyl group with resonances at  $\delta_{\text{C}}$  30.98 and  $\delta_{\text{H}}$  1.27 ppm was assigned as *pro-r*.

The other *gem* dimethyl system was assumed, therefore, to be attached to C23 ( $\delta_{\text{C}}$  70.39) and the methyl resonances at  $\delta_{\text{H}}$  1.13,  $\delta_{\text{C}}$  24.06 and  $\delta_{\text{H}}$  1.19,  $\delta_{\text{C}}$  27.48 ppm were arbitrarily assigned as C24 and C30 respectively. The carbon signal at  $\delta_{\text{C}}$  86.98 ppm showed XCORFE correlations with H24 and H30 and was therefore assigned to C22. This was supported by the HETCOR spectrum which indicated a single attached proton, the signal of which (at  $\delta_{\text{H}}$  3.71 ppm) was enhanced upon irradiation of the H24 methyl protons.

COSY correlations between H22 and  $\delta_{\text{H}}$  1.83 ppm and then between 1.83 to 1.68 ppm suggested the assignment of these resonances to the C21 and C20 protons respectively. Although the signals for H22 and H14 (see below) were almost coincident, they were readily resolved in the DQCOSY spectrum. The assignment of the resonance at  $\delta_{\text{H}}$  1.83 to the protons on C21 was further supported by nOe difference experiments. Irradiation of either the H24 methyl protons or the H22 proton gave enhancements of the signal at  $\delta_{\text{H}}$  1.83 ppm. A HETCOR correlation of  $\delta_{\text{H}}$  1.83 ppm with  $\delta_{\text{C}}$  26.11 ppm was observed. There were no other HETCOR peaks for  $\delta_{\text{C}}$  26.11 ppm, which together with the COSY data indicated that the proton resonances for C21 were coincident, or very nearly so.

The proton resonance at 1.68 ppm, tentatively assigned to one of the C20 protons, had a COSY correlation with a signal at  $\delta_{\text{H}}$  1.93 ppm, and both these signals had HETCOR correlations with  $\delta_{\text{C}}$  34.77 ppm. The XCORFE spectrum showed a correlation of C21 with  $\delta_{\text{H}}$  1.93 ppm. These data confirmed the assignment of C20 and its protons. The stereochemistry of these protons was assigned on the basis of

an nOe from H18 (see below) to the signal at  $\delta_{\text{H}}$  1.93 ppm which thus permitted the assignment of signal at  $\delta_{\text{H}}$  1.68 to the *pro-s* proton and  $\delta_{\text{H}}$  1.93 to the *pro-r*.

The second chemical shift based entry point into the assignment scheme was H18 at 4.9 ppm.<sup>101</sup> C18 was assigned from HETCOR data which showed a correlation of H18 with  $\delta_{\text{C}}$  78.41 ppm.

The assignment of C18 allowed the signals for the methyl group at C19 to be located, with XCORFE correlations of C18 and C20 with  $\delta_{\text{H}}$  1.2 ppm being observed. As there were three methyl groups with signals at 1.2 ppm it was not possible to decide which HETCOR and XCORFE correlations were due to C19 or C29. The assignment of the carbons signals at  $\delta_{\text{C}}$  84.11 and 23.02 ppm to C19 and C29 respectively, was made by difference after all the other signals had been assigned. The HETCOR and XCORFE data were consistent with these assignments.

C17 and its attached protons were assigned on the basis of the COSY and nOe connections from H18 to  $\delta_{\text{H}}$  1.68, and a HETCOR correlation with  $\delta_{\text{C}}$  20.59 ppm. COSY correlations of  $\delta_{\text{H}}$  1.68 with  $\delta_{\text{H}}$  1.25 and 1.57 ppm allowed the assignment of the protons on C16, and hence, the carbon signal ( $\delta_{\text{C}}$  32.08 ppm) which was located in the HETCOR spectrum. An XCORFE correlation of this carbon with  $\delta_{\text{H}}$  1.09 suggested an assignment for the methyl protons on C28. C28 ( $\delta_{\text{C}}$  23.04 ppm) was assigned from the HETCOR spectrum and an XCORFE correlation between  $\delta_{\text{H}}$  1.09 and  $\delta_{\text{C}}$  72.96 ppm indicated the assignment for C15.

These assignments for the C15 to C18 chain were consistent with XCORFE and nOe correlations from the H28 methyl protons to C14 and H14 respectively. Long range COSY and nOe connections between H18 and the protons on C16 were also observed.

Examination of a Courtauld model indicated the possibility that conformers existed for the C16-C17-C18 chain, such that either of the H16 protons could have

---

101 Williams, D.H. and Fleming, I., *Spectroscopic Methods in Organic Chemistry*, 2nd Edn., 133 (McGraw-Hill: Maidenhead, England 1973).



been responsible for the nOe observed to H18. A molecular mechanics study, described later in this section, was used to investigate this region of the molecule in an attempt to resolve this assignment.

The sharp singlet at  $\delta_{\text{H}}$  2.08 ppm is characteristic of the methyl protons of an acetate group.<sup>102</sup> The carbon resonance at  $\delta_{\text{C}}$  21.11 ppm was assigned from the HETCOR spectrum. These signals are absent from thyrsiferol spectra, which is consistent with this assignment.

Once the C15-C18 chain had been assigned only three methine carbons remained, C7, C11 and C14. The protons on these carbons were readily assigned by observing the nOe effects between the three remaining signals at  $\delta_{\text{H}}$  3.04, 3.55 and 3.68 ppm. Irradiation at  $\delta_{\text{H}}$  3.68 enhanced the signal at 3.55 ppm, as did irradiation at 3.04 ppm (see p72). Irradiation at  $\delta_{\text{H}}$  3.55 enhanced the signals at both 3.04 and 3.68 ppm. This implied that the resonance at  $\delta_{\text{H}}$  3.55 ppm was due to H11. An nOe at  $\delta_{\text{H}}$  3.68 ppm was also observed when the H28 methyl protons ( $\delta_{\text{H}}$  1.09) were irradiated which suggested that the signal at  $\delta_{\text{H}}$  3.68 was due to H14. Hence the H7 resonance was at  $\delta_{\text{H}}$  3.04 ppm. The carbon resonances at  $\delta_{\text{C}}$  86.50, 76.24 and 75.82 were correlated with H7, H11 and H14, respectively, in the HETCOR spectrum. The HETCOR correlation of  $\delta_{\text{H}}$  3.04 (H7) with  $\delta_{\text{C}}$  86.50 (C7), and the XCORFE correlation of  $\delta_{\text{C}}$  86.50 with the methyl protons at  $\delta_{\text{H}}$  1.19 (H26) confirmed that the assignment of H7 and H14 was correct. The presence of nOe enhancements between H7, H11 and H14 indicates that these three rings have the same conformation as in the crystal structure.

The assignment of H7, H11 and H14 now provided the basis for the assignment of the spectra for the remainder of the molecule.

COSY correlations were observed between H7 ( $\delta_{\text{H}}$  3.04 ppm) and signals at  $\delta_{\text{H}}$  1.41 and 1.74 ppm, which were therefore assumed to be the protons attached to C8.

---

102 Williams, D.H. and Fleming, I., *Spectroscopic Methods in Organic Chemistry*, 2nd Edn., 133 (McGraw-Hill : Maidenhead, England 1973).

Both these  $^1\text{H}$ -NMR signals showed HETCOR correlations with the carbon resonance at  $\delta_{\text{C}}$  22.94 ppm. These two protons, in turn, showed COSY correlations between  $\delta_{\text{H}}$  1.74 and 1.56 and between 1.41 and 1.78 ppm. There were HETCOR correlations of  $\delta_{\text{H}}$  1.56 and 1.78 with the carbon resonance at  $\delta_{\text{C}}$  38.53 ppm. The assignment of these signals to C9 and its associated protons was confirmed by an XCORFE correlation of  $\delta_{\text{C}}$  38.53 with  $\delta_{\text{H}}$  1.41 ppm.

The signal at  $\delta_{\text{H}}$  1.17 was tentatively assigned as being from the H27 methyl protons on the basis of an XCORFE correlation between  $\delta_{\text{C}}$  38.53 and  $\delta_{\text{H}}$  1.17 ppm. This was confirmed by the presence of an XCORFE correlation between  $\delta_{\text{H}}$  1.17 and C11 ( $\delta_{\text{C}}$  76.24). Examination of the HETCOR spectrum indicated that the C27 resonance was at  $\delta_{\text{C}}$  21.37 ppm. The C10 resonance was located at  $\delta_{\text{C}}$  71.87 ppm by an XCORFE correlation with the H27 signal at  $\delta_{\text{H}}$  1.17 ppm..

The assignment of the stereochemistry of the protons attached to C8 and C9 was made using nOe difference spectroscopy. Enhancement of the signals at  $\delta_{\text{H}}$  1.74 and 1.56 ppm was produced by irradiation of H7 ( $\delta_{\text{H}}$  3.04 ppm) which indicated the following assignments: H8r (equatorial),  $\delta_{\text{H}}$  1.74; H8s (axial)  $\delta_{\text{H}}$  1.41; H9r (equatorial),  $\delta_{\text{H}}$  1.78; H9s (axial),  $\delta_{\text{H}}$  1.56. A further nOe was observed for  $\delta_{\text{H}}$  1.78 when the H27 protons ( $\delta_{\text{H}}$  1.17 ppm) were irradiated.

The procedure used to assign the C12, C13 section was similar to that used for the C8, C9 fragment. C12 was assigned by observing the COSY correlations from H11 ( $\delta_{\text{H}}$  3.55 ppm) to  $\delta_{\text{H}}$  1.48 and 1.91, and the correlation, in the HETCOR spectrum, of these two protons with carbon signal  $\delta_{\text{C}}$  21.07 ppm. The stereochemical assignment was made from nOe data. Irradiation of H11 enhanced the signal at  $\delta_{\text{H}}$  1.91 ppm, thus suggesting that this proton was *pro-s*. An nOe from the H27 methyl protons to the signal at  $\delta_{\text{H}}$  1.48 ppm (*pro-r*) supported this proposal.

The carbon resonance for C13 was located at  $\delta_{\text{C}}$  23.80 ppm from the HETCOR spectrum, which showed a correlation for this signal with the signals for the attached protons, coincident, at  $\delta_{\text{H}}$  1.68 ppm. The latter assignment was indicated by COSY correlations from H12r and H12s to  $\delta_{\text{H}}$  1.68 ppm, and from H14. This

was further supported by the observation of an nOe at the  $\delta_H$  1.68 signal when the H28 methyl protons were irradiated.

The orientation of the two rings joined at C6-C7 was suggested by nOe experiments. Irradiation of H7 and of the H26 methyl protons, in turn, failed to produce a corresponding enhancement of the other signal. This implies that these two groups have the same *anti* orientation that is found in the crystal structure.

The nOe effects observed between the H14 and the H28 methyl protons indicated that the ring system is oriented differently from the crystal structure, where these two groups have an *anti* relationship along the C14-C15 bond and the H28 methyl protons are over 4Å from H14. Rotation energy calculations<sup>103</sup> for this bond indicated two possible minima, with rotations of 130° and 280° along C14-C15. In the first possible orientation is H14 2.3Å from the methyl group and the protons on C13 about 2.8Å away. In the second case the methyl group is sufficiently close to H14 (2.9Å), but protons on C13 are over 4.3Å distant.

The assignment of the C14-C19 chain was completed using the results of the series of molecular mechanics calculations described in the next section.

The  $^1H$  and  $^{13}C$ -NMR spectra of thyrsiferol were assigned by comparison with those of thyrsiferyl acetate. HETCOR and nOe data was used to resolve cases where the assignment was ambiguous.

### **Molecular Mechanics Calculations**

---

Chemical calculations that can predict the three dimensional structure and energies of molecules have been increasingly used by chemists to interpret experimental results and gain information that would be difficult to obtain by other methods. The methods of calculation may be divided into three categories: the *ab initio*

---

103 PCMODEL, Molecular Modelling Software for the IBM PC/XT/AT and Compatibles. (Serena Software : Bloomington, Indiana).

molecular orbital method, the semiempirical molecular orbital method, and molecular mechanics.

The molecular orbital methods build a representation of the molecular orbitals from the atomic orbitals of the constituents, such that the proper geometries are maintained and the electronic energy is minimised. The *ab initio* methods are the most basic with the treatment being largely derived from first principles. The semiempirical methods use a predetermined set of parameters to describe the shape and energy of the atomic orbitals. This approximation can make a more than fifty-fold improvement in the computation time required, but this is still at least an order of magnitude slower than molecular mechanics calculations. The computational time required for both these methods limits their use to simple molecules.<sup>104</sup>

Molecular mechanics is the only method suitable for a molecule the size of thyrsliferol. The molecular mechanics approach is purely empirical. The molecule is represented in a way that is analogous to a mechanical model composed of balls and springs. A series of potential energy functions are used to represent the stretching, bending and torsional deformations between bonded atoms, and the van der Waals forces between non-bonded atoms. The parameters of the potential energy functions are adjusted so that the molecular geometry, heats of formation, strain energy and other properties of molecules are reproduced. Since a large amount of experimental data is available it has been possible to generate parameter sets which give very accurate calculations, in many cases more accurate than the molecular orbital methods.

Molecular mechanics has some limitations. Firstly the calculations take no account of solvation or other intermolecular effects. Secondly, since the parameter sets are empirically derived the model may not work well with molecules with novel

---

104 Clark, T., *Handbook of Computational Chemistry* (Wiley-Interscience : New York 1985).

structural features. However the present programs, such as MM2 and MODEL used in this study, include many potential functions to ensure that the model performs well for a wide variety of molecules.<sup>105</sup>

#### Calculations for the C14 to C19 Chain of Thyrsiferyl Acetate

The only proton resonances that remained to be assigned were those of H16s and H16r. An nOe effect was observed between H18 and one of the H16 protons ( $\delta_H$  1.25 ppm), however, given the flexibility of the five bond chain from C14 to C19 this information was not sufficient to complete the assignment. To resolve this problem the C14-C19 chain was subjected to a conformational analysis using the program BAKMDL.<sup>106</sup> If the analysis showed a clear preference for conformers with one of the C16 protons closer to H18 than the other it would be then possible to use the nOe data to make the assignment. The method used here is similar to that used by Osawa *et al* to investigate the solution conformations of a series of tetra- and hexa-alditol peracetates,<sup>107</sup> and a series on bicyclo[3.3.1]nonan-9-one derivatives.<sup>108</sup> In the latter study the bulk dielectric constant in the electrostatic potential term was varied to take account of solvation effects and improve the agreement between the observed and calculated coupling constants. The effect of this variation is to reduce the electrostatic effects between non-bonded groups. This procedure was not possible in this study due to limitations in computer resources.

Using the crystal structure as a starting point, each of the five bonds in the chain was in turn rotated by 120° to generate all the possible non-eclipsed conformations,

---

105 Burkert,U. and Allinger,N.L., *Molecular Mechanics* (American Chemical Society : Washington 1982).

106 The program used, BAKMDL, is a derivative of the Still MODEL program, and was provided by Professor Kosta Steliou.

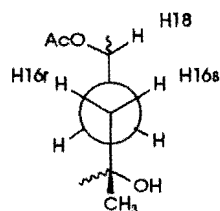
107 Masamune,S., Ma,P., Moore,R.E., Fujiyoshi,T., Jaime,C. and Osawa,E., *J. Chem. Soc., Chem. Comm.*, 261 (1986).

108 Jaime,C., Osawa,E., Takeuchi,Y. and Camps,P., *J. Org. Chem.*, **48**, 4514 (1983).

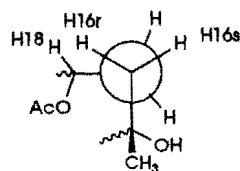
243 in total. Before any minimisation calculations were done the size of the computational task was reduced by eliminating conformers that had any non-bonded distances of less than 2.0 Å. After minimisation there were twenty-seven conformers which had MM2 energies within 3.0 kcal/mol of the minimum energy conformation.

The distances from H18 to the two hydrogen atoms on C16 were calculated, using XCONF,<sup>109</sup> and the values for the fifteen lowest energy conformers are shown on the following page. It was found that there were two groups of conformations. In the first group (group (a) in the diagram below), H16s in the calculation was approximately 2.4 Å from H18 while the other proton, H16r, was approximately 3.2 Å distant. In the second group of states, group (b), the situation was reversed with H16s being approximately 3.7 Å from H18 and H16r being approximately 2.7 Å away. Calculation of the populations of each state revealed that the first group of conformers accounted for seventeen of the twenty-seven possibilities and eighty percent of the population. The lowest energy conformation was found to be very close to the crystal structure, with the main difference being a slight change in the orientation of the furan ring. In the first group of conformers the C16-C17-C18 part of the chain was oriented such that the carbon-carbon bonds were *anti*, while the C15-C16 and C17-18 bonds were *gauche*.

The following table also shows the dihedral angles for the protons attached to C16, C17 and C18. The coupling constants were calculated and used to model the multiplet observed for C18 in the <sup>1</sup>H NMR spectrum. The coupling constant for



Group (a)



Group (b)

<sup>109</sup> The program XCONF is described later in this section.

## Selected Measurements from the Conformational Analysis of Thyrsiferyl Acetate

Conformer	%	$\Sigma\%$	MM2-E <sup>2</sup>	H18/H16 Distances (Å)		Dihedral Angles and Calculated Coupling Constants (Hz) <sup>1</sup>					
				H18-H16r	H18-H16s	H17r-H18	H17s-H18	H16s-H17s	H16s-H17r	H16r-H17s	H16r-H17r
1	26.0	26.0	78.22	3.26	2.44	179.9° (11.5)	-62.9° (1.9)	76.2° (0.7)	-166.7° (13.2)	-168.7° (13.4)	-51.6° (4.7)
2	23.9	49.9	78.27	3.24	2.44	179.7° (11.5)	-63.4° (1.9)	74.3° (1.0)	-168.7° (13.4)	-170.7° (13.6)	-53.7° (4.3)
3	10.8	60.7	78.74	3.23	2.40	179.4° (11.5)	-63.2° (1.9)	75.3° (0.8)	-168.3° (13.4)	-169.8° (13.5)	-53.3° (4.5)
4	9.3	70.0	78.83	2.73	3.83	75.8° (2.1)	-173.1° (11.6)	-15.5° (12.3)	96.6° (0.4)	98.2° (0.5)	-149.7° (10.3)
5	4.9	74.9	79.21	3.89	3.78	-61.5° (1.7)	52.6° (4.9)	71.0° (1.3)	-172.2° (13.7)	-172.4° (13.7)	-58.6° (3.3)
6	4.3	79.2	79.28	3.10	2.44	173.6° (11.1)	-71.4° (1.2)	70.9° (1.3)	-174.3° (13.8)	-176.0° (13.9)	-61.2° (2.9)
7	2.8	82.0	79.55	2.55	3.18	64.7° (3.4)	178.7° (11.5)	65.1° (2.2)	179.9° (13.9)	-179.2° (13.9)	-64.4° (2.0)
8	2.5	84.5	79.60	3.16	2.47	177.4° (11.4)	-66.0° (1.6)	70.5° (1.4)	-172.6° (13.7)	-174.2° (13.8)	-57.3° (3.6)
9	1.9	86.4	79.76	3.21	2.44	179.1° (11.5)	-64.6° (1.7)	73.4° (1.1)	-169.7° (13.5)	-172.8° (13.7)	-55.8° (4.0)
10	1.7	88.1	79.83	3.23	2.46	-178.7° (11.6)	-61.8° (2.0)	74.5° (1.0)	-168.3° (13.4)	-171.9° (13.7)	-54.6° (4.0)
11	1.4	89.5	79.95	3.14	2.49	178.6° (11.6)	-64.3° (1.6)	68.7° (1.6)	-174.2° (13.8)	-176.2° (13.9)	-59.1° (3.3)
12	1.3	90.8	80.00	3.08	2.45	174.8° (11.2)	-68.8° (1.4)	66.7° (1.9)	-176.5° (13.9)	-178.0° (13.9)	-61.2° (2.9)
13	1.2	92.0	80.06	2.91	3.86	80.0° (11.5)	-167.4° (11.5)	-92.2° (0.2)	75.5° (0.7)	76.7° (0.6)	-171.8° (13.7)
14	1.1	93.1	80.11	3.04	2.58	179.0° (11.6)	-64.3° (1.7)	58.4° (3.4)	175.2° (13.8)	173.7° (13.8)	-69.5° (1.4)
15	1.0	94.2	80.14	3.14	2.45	178.6° (11.6)	-64.6° (1.7)	68.3° (1.7)	-174.8° (13.8)	-176.4° (13.9)	-59.5° (3.1)

<sup>1</sup> Blunt, J.W., Macintosh Hypercard stack for the Karplus equation, using the method of Haasnoot, C.A.G., de Leeuw, F.A.A.M. and Altona, C., *Tetrahedron*, **36**, 2783 (1980).

<sup>2</sup> MM2 energies for each conformer in kCal/mol.

each of the spin interactions was factored by the fraction of the total population it represented then summed. Calculated coupling constants for H17r-H18 and H17s-H18 of 9.22Hz (observed 8.4Hz) and 3.2Hz (observed 5.2Hz) respectively were obtained. The agreement with the observed coupling constants in these calculations is similar to that obtained by Osawa *et al*, and supports the validity of this method of analysis.

Since an nOe effect was observed between H18 ( $\delta_H$  4.9ppm) and only one of the H16 resonances ( $\delta_H$  1.25ppm), and the molecular mechanics calculations showed a clear preference for conformations of the C14-C19 chain in which H16s was closer to H18 than H16r, the resonance at  $\delta_H$  1.25ppm was assigned to H16s and the remaining resonance ( $\delta_H$  1.57 ppm) was assigned as H16r.

In order to assess the influence of the orientation of the acetate group on the conformational analysis, the calculation was repeated, rotating the C18-OAc bond rather than the C14-C15 bond. Sixteen conformers within 3.0 kCal/mol of the lowest energy state were found. Eighty-six percent of the population was in conformations where the carbon-carbon bonds of C16-C17 and C17-C18 were *anti*, and where H16s was closer to H18 than H16r. Thus the orientation of the acetate would seem to have little influence on the orientation of the chain in this region.

### ***Software Written for Molecular Mechanics Analysis***

---

Three computer programs were written to aid the interpretation of the results of molecular mechanics calculations. The first, called BOLTZ, uses the Boltzmann distribution to calculate the populations of energy levels. The second, XGEOM, produces molecular geometry tables from a file of atomic position coordinates and the third, XCONF, generates comparative molecular geometry tables from the output of the conformational analysis program BAKMDL.

A further program, XLAT, was developed to translate Macintosh Chem3D files to a format that could be read by the molecular mechanics programs.



In each of these cases the programs reduced a tedious and potentially error prone task to a relatively straightforward procedure.

### BOLTZ

The program BOLTZ is used for calculating the populations of conformers given their energies. The energies for each level are entered, in kJ or kCal, and the relative populations of each energy level are displayed or printed.

The program evaluates the equation  $n_i = e^{-E_i/(k.T.N_A)}$  (where  $E_i$  is the energy in Joules for conformer  $i$ ,  $k$  is Boltzmann's constant in  $\text{JK}^{-1}$ ,  $T$  is the temperature in K and  $N_A$  is Avogadro's number) for each conformer, and the relative percentages of each conformer are calculated as  $n_i / \sum n_i \times 100$ .

A copy of the executable program (BOLTZ.EXE) and the Pascal source (BOLTZ.PAS) may be found in the disk at the back of this thesis, under the directory BOLTZ. The program may be used interactively, or with a prepared file such as TEST.ELS included on the disk. The program XCONF which analyses conformational analysis data writes a file that may be used in this way. Regardless of whether the calculations are printed, the results of the session are saved in a file called BOLTZ.TXT.

### XCALC

During the preparation of the of the *Delisea* paper<sup>110</sup> (see next section) it became apparent that the publication tables of bond lengths and angles, prepared by the SHELX suite of programs were incomplete. Recalculation of these tables required

---

110 McCombs, J.D., Blunt, J.W., Chambers, M.V., Munro, M.H.G. and Robinson, W.T., *Tetrahedron*, **44**, 1489 (1988).

the original reflection data which no longer existed for the structures that were solved prior to the ones described in this thesis. The only data available were the atomic position coordinates and temperature and their estimated standard deviations (ESD's). To solve this problem the program XCALC was written to generate tables of bond lengths and bond angles, with ESD's,<sup>111,112,113</sup> from the available data.

The directory XCALC on the disk contains a copy of executable (XCALC.EXE) and Pascal source (XCALC.PAS) versions of the program. A SHELX input file for Eudistomin K p-bromobenzoate is included as test (RJL1.INP).

### **XGEOM**

The program XGEOM, a derivative of XCALC, was developed as a more generally applicable program to produce molecular geometry tables from atomic position data obtained from sources such as published material and molecular mechanics calculations. XGEOM does no calculations of estimated standard deviation's (ESD's), nor does it include tables of thermal parameters. However it does produce a table of torsion angles.

The input file is similar to that for XCALC, but without the temperature parameters and ESD's. The first two lines in the input file are SHELX format "TITL" and "CELL" instructions, and the last line must be an "END" instruction. There is one line per atom, and only four parameters are required: the atom label; the atom type, which is ignored; the fractional atomic coordinates.

The executable (XGEOM.EXE) and Pascal source (XGEOM.PAS) versions of the program are in the XGEOM directory on the floppy disk at the back of this thesis.

---

111 Ladd, M.F.C. and Palmer, R.A., *Structure Determination by X-ray Crystallography* (Plenum Press : New York 1977).

112 Rollett, J.S. (Ed), *Computing Methods in Crystallography* (Pergamon Press : Oxford 1965).

113 Darlow, S.F., *Acta Cryst.*, 683 (1960).

The file RJL1.DAT is an input file with the data for one molecule of eudistomin K *p*-bromobenzoate. If a disk file is selected for output it will have the same name as the input file, with the extension .GEO.

## XCONF

XCONF was developed, from XCALC, as an aid to the interpretation of the often substantial output from the molecular mechanics conformation analysis program BAKMDL.

BAKMDL generates two output files, one containing the structures of the conformers that were found to have minimised energies within 3.0 kCal/mol of the lowest energy structure, and the other a printer output file, containing, amongst other data, the energy of each conformer.

XCONF reads the MODEL format structure file (.DAT), and the printer listing file (.BKM) written by BAKMDL to produce a file (with the extension .LST) that contains molecular geometry tables of selected atoms for each structure.

Each line in the output file is prefixed by a key which enables the file to be sorted such that measurements for a particular length or angle from each conformer are placed together. Either the measurement or the energy may be included in the key so that within each measurement group the entries will be ordered in terms of increasing value of the measurement or the energy.

The format of the key is  $x/y1/y2/y3/y4/v/c$  where  $x$  is the table type (1=atomic position coordinates, 2=bond lengths, 3=bond angles, 4=torsion angles),  $y1..y4$  are the atom numbers involved,  $v$  is either the measurement or the energy, and  $c$  is the conformer number. For example, a bond angle ( $108.93^\circ$ ) for C3-C9-C10 in conformation four would be coded 3/003/009/010/000/00108.93/004.

The output file produced by XCONF contains a series of tables, one for each conformer found in the BAKMDL file, which must then be sorted using the PC-DOS sort program to aggregate the data for each length or angle. The table

below shows the output produced from the test data when atom 23, 24, 25 and 26 were selected.

Table 3. Bond Angles (deg.)					
3/023/024/025/000/0112.665/001	A(23)	- A(24)	- A(25)	112.7	78.220
3/024/025/026/000/0115.753/001	A(24)	- A(25)	- A(26)	115.8	78.220
Table 3. Bond Angles (deg.)					
3/023/024/025/000/0112.472/002	A(23)	- A(24)	- A(25)	112.5	78.270
3/024/025/026/000/0115.868/002	A(24)	- A(25)	- A(26)	115.9	78.270
Table 3. Bond Angles (deg.)					
3/023/024/025/000/0112.733/003	A(23)	- A(24)	- A(25)	112.7	78.740
3/024/025/026/000/0116.472/003	A(24)	- A(25)	- A(26)	116.5	78.740
Table 3. Bond Angles (deg.)					
3/023/024/025/000/0112.198/004	A(23)	- A(24)	- A(25)	112.2	78.830
3/024/025/026/000/0116.850/004	A(24)	- A(25)	- A(26)	116.9	78.830

The DOS command "SORT <TEST.LST >TEST.TXT" was used to sort the file TEST.LST (produced by XCONF) to the file TEST.TXT, which is shown below. The entries for each angle are now grouped together, and sorted within each group so that conformers with similar measurements are placed together within each section.

3/023/024/025/000/0112.198/004	A(23)	- A(24)	- A(25)	112.2	78.830
3/023/024/025/000/0112.472/002	A(23)	- A(24)	- A(25)	112.5	78.270
3/023/024/025/000/0112.665/001	A(23)	- A(24)	- A(25)	112.7	78.220
3/023/024/025/000/0112.733/003	A(23)	- A(24)	- A(25)	112.7	78.740
3/024/025/026/000/0115.753/001	A(24)	- A(25)	- A(26)	115.8	78.220
3/024/025/026/000/0115.868/002	A(24)	- A(25)	- A(26)	115.9	78.270
3/024/025/026/000/0116.472/003	A(24)	- A(25)	- A(26)	116.5	78.740
3/024/025/026/000/0116.850/004	A(24)	- A(25)	- A(26)	116.9	78.830

The atom numbers to be included in the analysis must be entered, and only those atoms which were specified will appear in the table. The program allows any combination of bond length, bond angle and torsion angle tables to be selected. Hydrogen atoms may be omitted.

A second file, with the extension .ELS, is also produced. This file contains the energy level data from the BAKMDL listing file, and is in a format suitable for use with energy level population calculation program BOLTZ. The DOS command "BOLTZ <TEST.ELS" directs BOLTZ to use the file TEST.ELS as a substitute for keyboard input.

The XCONF directory on the disk at the back of this thesis contains executable (XCONF.EXE) and Pascal source (XCONF.PAS) versions of the program. The files TEST.DAT and TEST.BKM contain test data.

### ***XLAT - Translation of Chem3D files to MMX Format***

Two commercial computer packages were used to manipulate three dimensional representations of molecules in the work described in this thesis. The Macintosh molecular graphics program Chem3D, provided a simple and flexible method of drawing high-quality three dimensional views of molecules, while the suite of molecular mechanics programs, based around MM2, was used for conformational analysis.

While the molecular mechanics program MODEL could write files in a format for Chem3D, transfer of atomic position data from Chem3D to MODEL required a very tedious hand conversion of the data to MM2 or MMX format (the format of the MODEL input file was not available). For a molecule with thirty or forty atoms this was not a trivial exercise.

The biggest difference between the two systems was the method of representing the connections between atoms. The Chem3D cartesian file described the connectivity with a list of nearest neighbours for each atom. For example 3-methyl-pentane would be described thus:

```
C1 1
C2 1 3
C3 2 4 6
C4 3 5
C5 4
```

For MMX the connectivity is represented in terms of unique chains of connected atoms, with any atoms not included in a chain being described as connected pairs. 3-Methyl-pentane could be described by two chains, 1-2-3-6 and 3-4-5 or by one chain, 1-2-3-4-5 and one connected pair 3-6.

To enable molecular mechanics to be performed routinely on Chem3D structures the program XLAT was written to do the conversion automatically. In addition MMX files may be converted to Chem3D format for the convenience of PC-MODEL users. Presently the conversion program does not include insertion of lone-pairs, nor does it deal with double bonds (with the exception of carbonyl groups) as this information is not present in the Chem3D cartesian file. The range of

atom types converted is at present limited to carbon, hydrogen, nitrogen, sulphur and oxygen.

The procedure used to generate the connectivity chains is as follows. Taking each atom in turn all possible trees from each atom are generated (using a recursive algorithm) to find the longest. The longest tree is written to the file, and the bonds used are removed from the list of those available. This process is repeated until thirty chains have been generated, or no chains of more than two atoms remain.

The unconnected atoms are then attached as connected pairs.

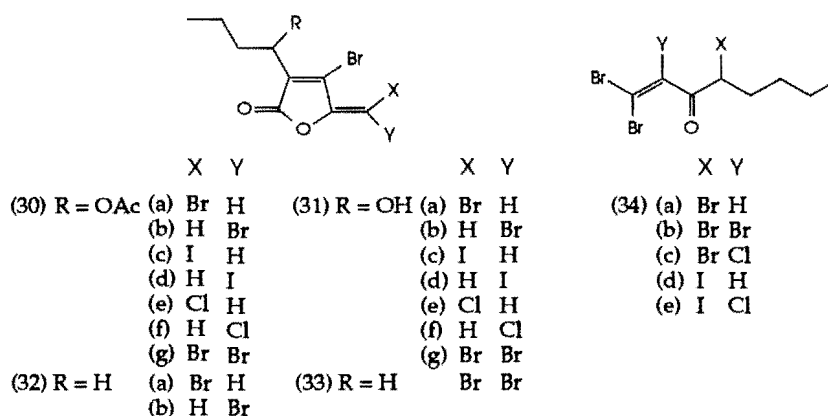
When XLAT is run it asks for the type of conversion to be done, and the name of the file, entered without an extension. Chem3D cartesian files are expected to have the extension .C3D and MMX files .MMX.

The executable file XLAT.EXE and the Pascal source (XLAT.PAS) may be found in the sub-directory XLAT on the diskette at the back of this thesis.

### *Butenolides from Delisea elegans*

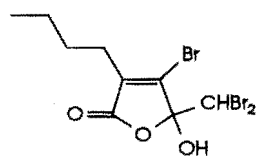
A wide range of halogenated metabolites,<sup>114</sup> including butenones, acetones, acrylic and acetic acids, pyranones and octenones<sup>115,116,117,118</sup> have been reported from marine red algae of the family *Bonnemaisoniaceae*. Of particular note, both for their antimicrobial activity and their unusual structure, are a number of halogenated 2(5H)-furanones, named fimbrolides, which have been isolated from species of the genus *Delisea*.

The structures (30) through (33)<sup>119</sup> were isolated from the dichloromethane soluble material of extracts of *D. fimbriata*, collected on the east coast of Australia. A similar

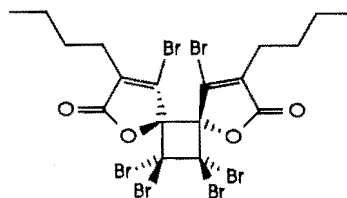


- 114 McConnell, O.J. and Fenical, W., *Antimicrobial Agents from Red Marine Algae of the Family Bonnemaisoniaceae in Marine Algae Pharmaceutical Science*, 403 (Eds Hoppe, H.A., Levring, T. and Tanaka, Y.) (de Gruyter: Berlin 1979).
- 115 Woolard, F.X. and Moore, R.E., *Tetrahedron*, **32**, 2843 (1976).
- 116 McConnell, O.J. and Fenical, W., *Phytochemistry*, **16**, 367 (1977).
- 117 Burreson, B.J., Moore, R.E. and Roller, P., *Tetrahedron Lett.*, 473 (1975).
- 118 Kazlauskas, R., Lidgard, R.O. and Wells, R.J., *Tetrahedron Lett.*, 3165 (1978).
- 119 Kazlauskas, R., Murphy, P.T., Quinn, R.J. and Wells, R.J., *Tetrahedron Lett.*, 37 (1977).

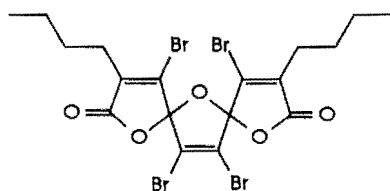
study of *D. fimbriata* collected from Antarctica described the isolation of 2(5H)-furanones (30a) - (30d), (30f) and (30g),<sup>120</sup> together with a series of oct-1-en-3-ones (34a) - (34e).<sup>121</sup> In both studies interest in the species was due to observations of biological activity. In the first instance high *in vitro* antimicrobial activity was found, while in the second, plants were observed to be remarkably free of epibionts. Three further compounds (35), (36),<sup>122</sup> (37) and (38),<sup>123</sup> each related to (33), were isolated from *D. elegans* collected in New Zealand.



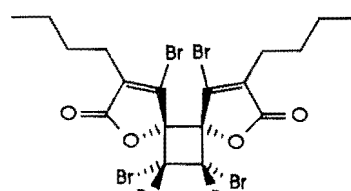
(35)



(36)



(37)



(38)

In this study two new metabolites (39) and (40) were isolated from *Delisea elegans* collected at Kaikoura, New Zealand.<sup>124</sup> The least polar compound in the extracts was heptadecane, which was identified by GC and by <sup>13</sup>C-NMR. The major component of the extracts, also among the least polar of the compounds, was the previously reported furanone (33). This compound was estimated to represent 80% of the organic soluble material in each of the extracts. The two new compounds

120 Pettus, J.A., Wing, R.M. and Sims, J.J., *Tetrahedron Lett.*, 41 (1977).

121 Rose, A.F., Pettus, J.A. and Sims, J.J., *Tetrahedron Lett.*, 1847 (1977).

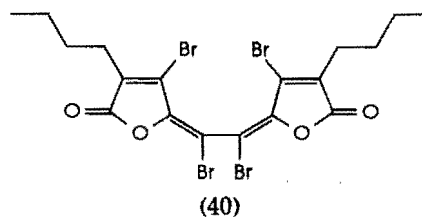
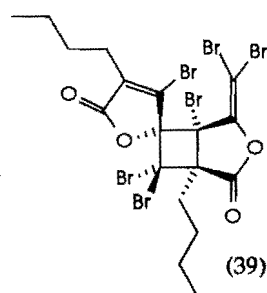
122 McCombs, J.D., MSc Thesis (University of Canterbury : Christchurch 1982).

123 Crystal structures of compounds (37) and (38) isolated in the study detailed in the previous reference were determined by Chambers, M.V. and Malvinas, P. as part of BSc (Hons) projects.

124 McCombs, J.D., Blunt, J.W., Chambers, M.V., Munro, M.H.G. and Robinson, W.T., *Tetrahedron*, 44, 1489 (1988).

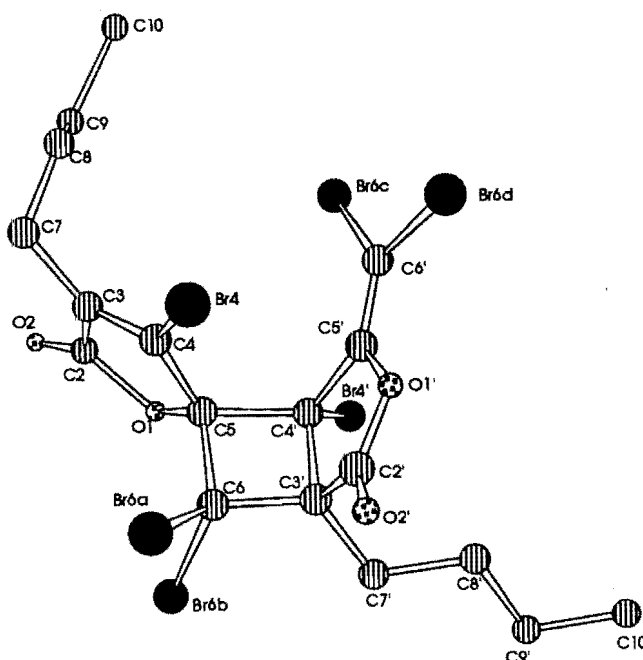


isolated were characterised by single crystal X-ray structure determinations as (Z,Z)-5,5'-(1,2-dibromo-1,2-ethanediylidene)bis[4-bromo-3-butyl-2(5H)-furanone] (40) and 3,5',7',7'-tetrabromo-1',4'-dibutyl-4'-(dibromomethylene)spiro[furan-2(5H), 6'-[3]oxabicyclo[3.2.0]heptane]-2',5-dione (39).



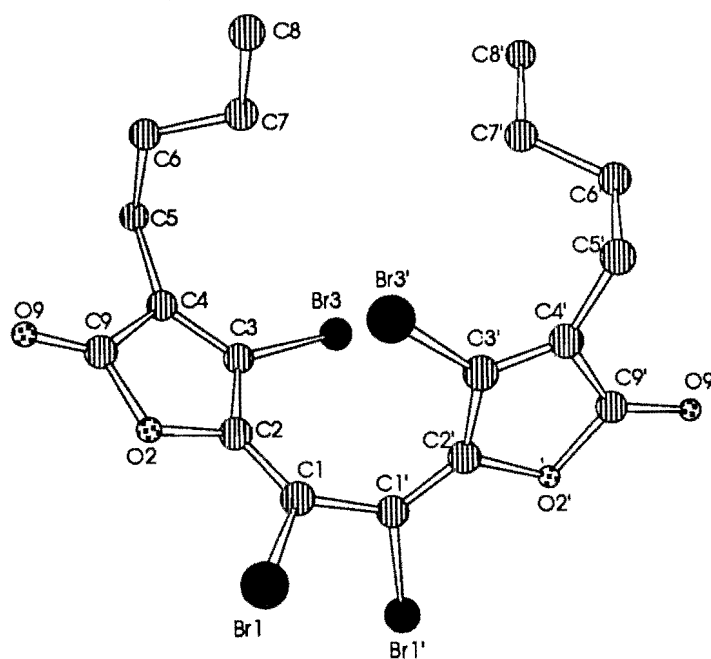
Crystals suitable for single crystal X-ray diffraction studies were obtained by recrystallisation of these compounds (39) and (40) from ether/pet. ether. The cell parameters were determined by the least-squares refinement of the setting angles of twenty-five accurately centred high angles reflections. The space group, P1 in each case, was initially indicated by systematic absences of the appropriate reflections and subsequently confirmed as a result of the structure analysis. Perspective drawings of the compounds are shown below.

The bromine atom positions were found by direct methods, and the remaining non-hydrogen atoms were located from difference Fourier syntheses.



Blocked-cascade least-squares refinements were employed to minimise the function  $\sum w(|F_o| - |F_c|)^2$  with reflection weights of  $1/[\sigma^2(F) + g(F^2)]$ .<sup>125</sup> All non-hydrogen atoms were assigned anisotropic thermal parameters, and the hydrogen atoms were added at calculated positions as rigid groups pivoting about their carbon atoms. Numerical absorption corrections were used in each case. The final electron density maps showed no residual electron density, and there were no abnormal discrepancies between observed and calculated structure factors.

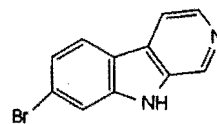
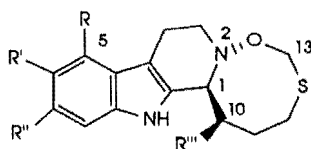
The origin of these unusual compounds is unclear. Some attempts were made to form the dimeric compounds by photolysis of the butenolide (33), however <sup>13</sup>C-NMR gave no indication of the presence of any of the dimers, and no pure compounds were isolated. Another member of the marine chemistry group at the University of Canterbury is pursuing this work.



125 Sheldrick, G.M., *SHELXTL User Manual*, Revision 4.1 (Nicolet XRD Corporation: Cupertino, CA 1981).

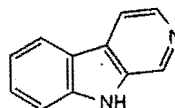
## Crystal Structure of Eudistomin K

The eudistomins are a series of  $\beta$ -carboline derivatives which were first isolated from the Caribbean ascidian *Eudistoma olivaceum* (sub-order Aplousobranchia, family Polycitoridae).<sup>126,127,128</sup> These compounds are of considerable interest for their potent *in vitro* antiviral activity, and for the unprecedented seven-membered oxathiazepine ring found in eudistomins C, E, F, K and L, (41a) - (41e). More recently the eudistomins were found to be responsible for the *in vitro* antiviral and antitumour activity of the New Zealand ascidian *Ritterella sigillinoides* (family



(42)

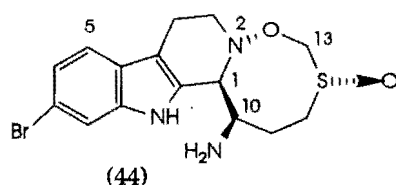
		R	R'	R''	R'''
(41)	C (a)	H	OH	Br	NH <sub>2</sub>
	E (b)	Br	OH	H	NH <sub>2</sub>
	F (c)	H	OH	Br	NHC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>
	K (d)	H	H	Br	NH <sub>2</sub>
	L (e)	H	Br	H	NH <sub>2</sub>
	(f)	H	H	H	NH <sub>2</sub>
	(g)	H	H	Br	NH-p-Br-Bz
	(h)	H	H	Br	+NH <sub>3</sub>
	(i)	H	H	Br	NHCOCH <sub>3</sub>



(43)

Polyclinidae). Eudistomins K, C and O (42) were isolated, together with  $\beta$ -carboline (43) and two new compounds, eudistomin K sulfoxide (44) and debromoeudistomin K (41f).<sup>129,130,131</sup>

126. Rinehart, K.L. Jr., Kobayashi, J., Harbour, G.C., Hughes, R.G. Jr., Mizsak, S.A. and Scatell, T.A., *J. Am. Chem. Soc.*, **106**, 1524 (1984)
127. Kobayashi, J., Harbour, G.C., Gilmore, J. and Rinehart, K.L. Jr., *J. Am. Chem. Soc.*, **106**, 1526 (1987).
128. Rinehart, K.L., Kobayashi, J., Harbour, G.C., Gilmore, J., Mascall, M., Holt, T.G., Sheild, L.S. and Lafargue, F., *J. Am. Chem. Soc.*, **109**, 3378 (1987).
129. Blunt, J.W., Lake, R.J., Munro, M.H.G., and Toyokuni, T., *Tetrahedron Lett.*, **28**, 1825 (1987).
130. Lake, R.J., Brennan, M.M., Blunt, J.W., Munro, M.H.G. and Pannell, L.K., *Tetrahedron Lett.*, **29**, 2255 (1988).
131. Lake, R.J., Blunt, J.W., Munro, M.H.G., *Aust. J. Chem.*, in press.



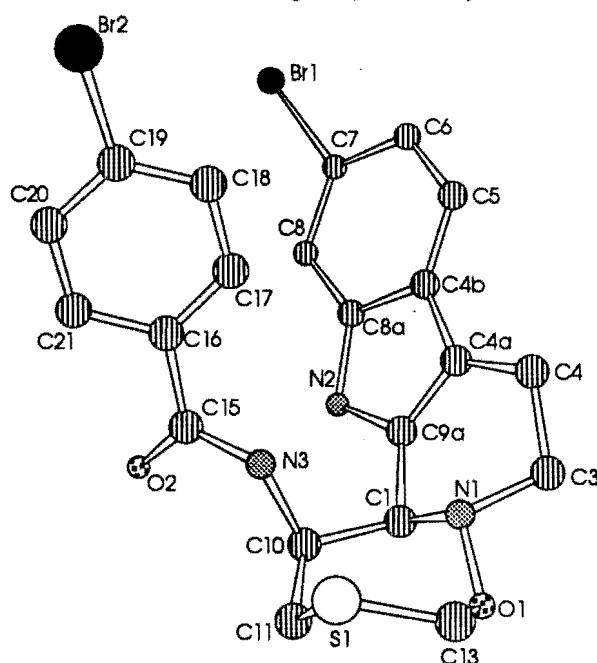
The original structural assignments of the eudistomins by Rinehart *et al* were largely based on NMR and mass spectrometric evidence and the absolute configuration at C1 was established from the CD spectrum. The subsequent extensive NMR study, of Blunt *et al*, of eudistomin K (41d) used a modified Karplus equation was used to estimate dihedral angles in the oxathiazepine and  $\beta$ -carboline rings. This geometric data together with selected nOe measurements showed that the relative stereochemistry of the N-O bond was  $2\alpha$  not  $2\beta$  as suggested by Rinehart *et al*. The solution conformation for the eudistomin K was also proposed on the basis of this data. Although the spectroscopic data from these studies strongly supported the proposed oxathiazepine ring, it was considered desirable to determine the X-ray crystal structure of one of these compounds.

In this study the X-ray crystal structure and absolute configuration of the *p*-bromobenzoyl derivative of eudistomin K (41g) was determined.<sup>132</sup> It was found that the conformation of the crystal structure was only slightly different from that proposed for the solution conformation of eudistomin K (41d). The coupling constants and nOe relationships measured for eudistomin K (41d), the salt (41h) and the acetyl (41i) and *p*-bromobenzoyl (41g) derivatives are similar, indicating the same dominant conformation for the oxathiazepine ring, regardless of the bulk and polarity of the substituent at C10. These observations strongly suggest that the factors determining the the solution and crystal conformations of eudistomin K (41c) and its derivatives are essentially the same. A perspective drawing of eudistomin K *p*-bromobenzoate (41g) is shown below.

---

132. Lake, R.J., McCombs, J.D., Blunt, J.W., Munro, M.H.G. and Robinson, W.T., *Tetrahedron Lett.* 29, 4971 (1988).

Eudistomin K (41d) was converted to the *p*-bromobenzoyl derivative (41g) by standard methods.<sup>133</sup> Recrystallisation from methanol/chloroform gave long, thin, tabular, transparent, needles suitable for single crystal X-ray structure analysis.



To guard against the possibility that the compound was unstable in air or the X-ray beam, the data set was initially collected rapidly using Wyckoff scans. Many attempts were made using both Patterson and direct methods to find a phasing model. Atomic position coordinates were calculated for the *p*-bromobenzoate and the bromoindole groups, using the PCMODEL molecular mechanics program.<sup>134</sup> This data was used to perform rotational and translational searches of Patterson space (PATSEE),<sup>135</sup> but without success. Unfortunately the crystal selected for the X-ray analysis was extremely thin. This factor combined with the fast data collection resulted in data which were too poor to allow the analysis to proceed

133. The *p*-bromobenzoate was prepared using *p*-bromobenzoyl chloride/triethylamine and recrystallised by Dr R.J. Lake.

134. PCMODEL, Molecular Modelling Software for the IBM PC/XT/AT and Compatible, (Serena Software : Bloomington, Indiana).

135. Egert, E. and Sheldrick, G.M., *Acta Cryst.*, **A41**, 262 (1985).

beyond locating the bromine atom positions from a Patterson synthesis (SHELXS).<sup>136</sup>

A second data collection was then undertaken. An exhaustive search of all the available crystalline material provided a single crystal which was thicker than the original. Data were collected using  $\omega$ -scan intensity measurements, and of the 4374 unique reflections, 3009 were judged observed ( $I > 3\sigma(I)$ ). Check reflections were measured every 500 and no significant variations occurred. Corrections were made for Lorentz and polarisation effects, and an empirical absorption correction based on  $\psi$  scan data was applied.

A Patterson calculation revealed the positions of the bromine and sulphur atoms. The remaining non-hydrogen atoms were located from difference Fourier syntheses (SHELXTL).<sup>137</sup> The asymmetric unit was found to contain two independent eudistomin K molecules and one methanol molecule. The two eudistomin molecules were conformationally equivalent, their slight differences being due to different packing environments.

The data were too poor to allow anisotropic refinement of the complete molecule. The carbon atoms of the indole group remained isotropic, while all other atoms were permitted to refine anisotropically.

All hydrogen atoms were inserted at calculated positions. The only exception was for the hydrogen on the methanol oxygen atom which is potentially able to hydrogen bond to several sites. Since this hydrogen atom could not be located in difference Fourier syntheses it was omitted. The indolic hydrogen atom, H(2), was inserted in the plane of the ring. The length of the N(3) - C(15) bond, 1.35 Å, indicated a degree of double bond character, hence the H(3) hydrogen atom was inserted in the plane defined by C(15), N(3) and C(10).

---

136. Sheldrick, G.M., *SHELXS. Program for Crystal Structure Solution* (University of Gottingen, Federal Republic of Germany).

137. Sheldrick, G.M., *SHELXTL User Manual*, Revision 4.1 (Nicolet XRD Corporation: Madison, Wisconsin, U.S.A 1981).

Blocked-cascade (SHELXTL) refinements of 451 least squares parameters converged with  $R = 0.049$  and  $wR = 0.053$ . The final electron density maps showed no significant residual electron density and there were no abnormal discrepancies between observed and calculated structure factors.

The absolute configuration is often obtained as a by-product of normal data collection. Commonly, the ratio of the  $R$  factors for two enantiomeric models, over all the collected data, are compared using Hamilton's ratio test to assess the statistical significance. The method is readily applied, but in this case gave ambiguous results. An alternative procedure, *eta* refinement, was therefore used to unambiguously determine the absolute configuration.<sup>138</sup> Instead of inverting the structure, which for many cases involves a change of space group, the sign of the atomic scattering factor  $if_j''$  term for the anomalous scatterers may be reversed to obtain the two values of  $R$ . In the method used here a chirality parameter  $\eta$  is added to the give  $\eta if_j''$  as the anomalous scattering factor term. The value of  $\eta$  is set to starting values of +1 then -1 and allowed to refine. The parameter should converge, in each refinement, to a value close to +1 or -1, indicating whether the current model or its enantiomer is correct. The value of  $\eta$ , and its estimated standard deviation, obtained in this study, +0.95(3), was decisive.

Fractional atomic coordinates for both eudistomin K *p*-bromobenzoate (41g) molecules and the methanol solvate molecule are listed in the appendices.

Further direct evidence confirming the structure for the oxathiazepine eudistomins, and their absolute configuration, has recently been provided by the total synthesis of eudistomin L (41e).<sup>139</sup>

---

138. Rogers, D., *Acta Cryst.*, **A37**, 734 (1981).

139. Nakagawa, M., Jin-Jun Liu and Hino, T. *J. Am. Chem. Soc.*, **111**, 2721 (1989).

## Crystal Structure of Discorhabdin C

---

Sponges of the genus *Latrunculia* du Bocage (order Hadromerida, family Latrunculiidae) occur as rather striking green or red-brown domes in deep water (>15m), around the New Zealand coastline. Apart from lipids, the only reported metabolites from a *Latrunculia* species, are the latrunculins.<sup>140</sup> These ichthyocytotoxic compounds were isolated from the tropical species *L. magnifica*. It has been suggested, however, that this species has been incorrectly placed in the *Latrunculia* genus.<sup>141</sup>

Extracts of these sponges were found to have high *in vitro* cytotoxic activity. A bio-assay directed analysis of the extract of an undescribed *Latrunculia* species by reverse-phase flash chromatography and HPLC yielded a highly cytotoxic (ED<sub>50</sub> <100ng/ml for L1210 tumour cells) red-brown compound named discorhabdin C (45).<sup>142</sup> In this study the structure of this compound was determined by single crystal X-ray crystallography.<sup>143</sup> This result provided the basis for the spectroscopic determination of the structure of discorhabdins A (46), B (47),<sup>144</sup> D (48)<sup>145</sup> and a number of synthetic derivatives.<sup>146</sup> Discorhabdins have also been reported from sponges of the genus *Prianos*.<sup>147,148</sup>

---

140 Kashman, Y., Groweiss, A., Lidor, R., Blasberger, D. and Carmely, S., *Tetrahedron*, **41**, 1905 (1985).

141 Bergquist, P.R., Personal communication to Dr N.B. Perry.

142 The isolation, crystallisation and spectroscopy were performed by Dr N.B. Perry.

143 Perry, N.B., Blunt, J.W., McCombs, J.D and Munro, M.H.G., *J. Org. Chem.*, **51**, 5478 (1986).

144 Perry, N.B., Blunt, J.W. and Munro, M.H.G., *Tetrahedron*, **44**, 1727 (1988).

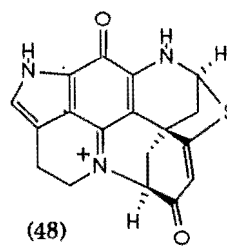
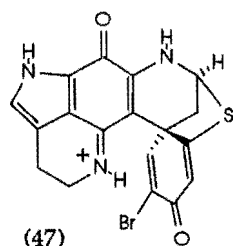
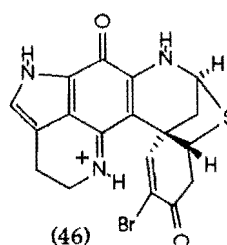
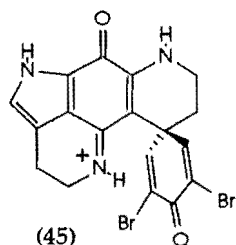
145 Perry, N.B., Blunt, J.W., Munro, M.H.G., Higa, T. and Sakai, R., *J. Org. Chem.*, **53**, 4127 (1988).

146 Copp, B.R., personal communication.

147 Cheng, J., Ohizumi, Y., Wälchli, M.R., Nakamura, H., Hirata, Y., Sasaki, T. and Kobayashi, J., *J. Org. Chem.*, **53**, 4621 (1988).

148 Kobayashi, J., Cheng, J., Isibashi, M., Nakamura, H., Ohizumi, Y., Hirata, Y., Sasaki, T., Lu, H. and Clardy, J., *Tetrahedron Lett.*, **28**, 4939 (1987).

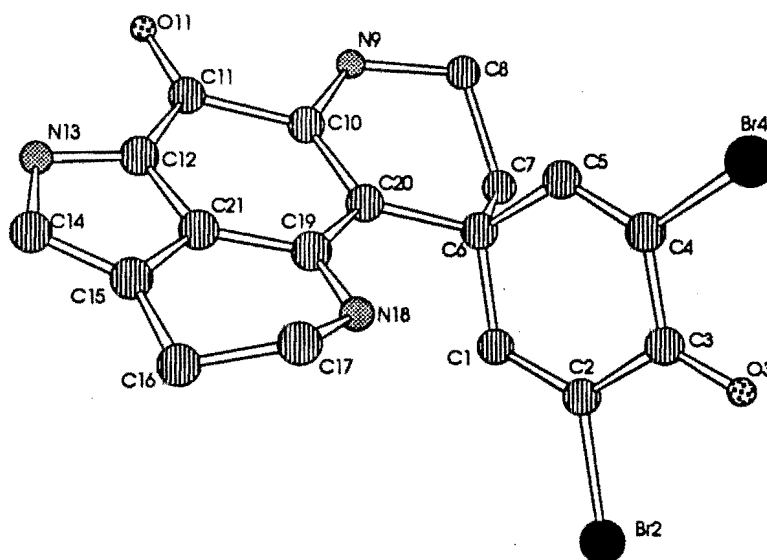




Although spectroscopic methods yielded considerable information about discorhabdin C, the structure could not be determined. Crystals suitable for a single crystal X-ray diffraction study were obtained by recrystallisation from water/methanol. Precession photography of a small crystal indicated a triclinic system. The accurate cell constants were determined by a least-squares fit of twenty-three high angle reflections ( $a$  8.470(2)Å,  $b$  10.562(2)Å,  $c$  13.810(2)Å,  $\alpha$  67.84(1)°,  $\beta$  78.53(1)°,  $\gamma$  88.38(2)°). The choice of space group,  $P1$ , was confirmed by the successful structure determination. All unique diffraction maxima with  $2\theta < 50^\circ$  were collected at 150K. Variable speed  $\omega$  scans were used with graphite monochromated Mo  $K\alpha$  radiation (0.7107Å). Corrections for background, Lorentz and polarization effects were applied to the 3356 unique reflections measured. The 2878 reflections judged to be observed,  $I > 3\sigma(I)$ , were used for the structure solution and subsequent refinement of the structural parameters.

Standard heavy atom methods were used for the structure solution. The positions of the bromine atoms were obtained from the Patterson synthesis, and the remaining non-hydrogen atoms were located in subsequent difference Fourier maps. Initial ambiguities in the scattering factor assignments were resolved with the aid of spectroscopic data, and by analysis of the behaviour of the temperature factors during the refinement calculations. Hydrogen atoms were added at calculated positions. After applying an empirical absorption correction, a full least

squares refinement, with anisotropic non-hydrogen atoms, converged to a standard crystallographic R factor of 0.055.<sup>149</sup> Two independent discorhabdin C molecules, two trifluoroacetate and six water molecules were found in the unit cell.



A perspective drawing of a single molecule of protonated discorhabdin C is shown above. The iminoquinone chromophore is almost planar, while the two six-membered heterocyclic rings are in half boat conformations. The bond lengths of N9-C10 and N18-C19 are both about 1.33Å, indicating a similar degree of double bond character and delocalisation of the positive charge between the two nitrogens. The dibromocyclohexadienone ring is "pushed away" from N18 with the C3-C6-C20 angle being 23° larger than that for C3-C6-C7 and the plane of the dibromocyclohexadienone ring is at an angle of 76° to the plane of the iminoquinone moiety. This results in considerable strain at the *spiro* junction, C6, with bond angles ranging from 104° to 113° and an unusually long bond of 1.58Å at C6-C7.

The pyrrolo[1,7]phenanthroline skeleton of discorhabdin C represents a new class of cytotoxic compounds. Another member of this research group has devoted considerable effort to synthesising derivatives in an effort to enhance the biological

149 All calculations were performed on a DG Nova 4X using SHELXTL: Sheldrick, G.M., *SHELXTL User Manual*, Revision 4 (Nicolet XRD Corporation : Cupertino, CA 1983).

activity and to understand the structure/activity relationships for these compounds.

## ***MassCalc - A Mass Spectrometry Calculator***

---

Interpreting mass spectra typically involves a considerable amount of tedious arithmetic analysis, which when done manually is potentially error prone and diverts attention from the task of solving the problem at hand. However, the procedures which are usually carried out with a calculator and a table of atomic masses are readily automated. Computer programs to assist the interpretation process are commonly included in mass spectrometer data systems, but they are often inaccessible and impenetrably complex to the casual user. The program "MassCalc" was developed, over several years, to meet the need for a readily available and simple to use tool for the organic chemist. The goal was to produce an intelligent calculator to reduce the effort required by the chemist, rather than to automatically interpret spectra. Other similar programs have since appeared.<sup>150</sup> MassCalc was used extensively to interpret the spectra obtained in the course of the work described in this thesis, and has been used by other members of the Chemistry Department Marine group.

An important criterion in the design of the program was ease of use. While a menu driven approach provides the greatest assistance to the novice user, this type of interface quickly becomes a hindrance when the user is more familiar with the program. For this reason a system of entering two character commands, followed by the appropriate parameters, was chosen. Either complete or partial commands may be entered. The program then prompts the user for the missing parts. Extensive "help" screens were provided to explain the commands. The present version makes limited use of a mouse to label peaks in the spectrum plots.

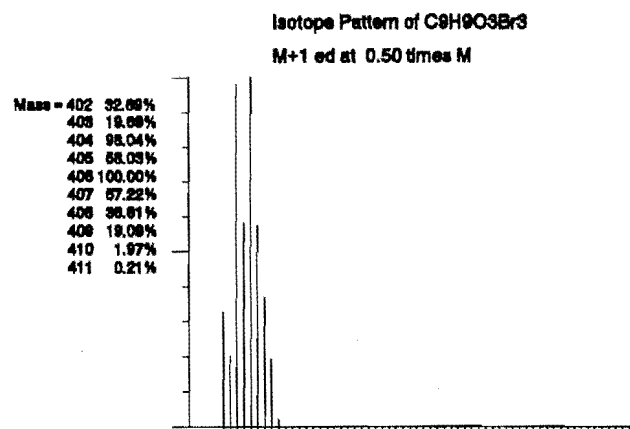
Initial versions of MassCalc were written on a PDP-11 computer in standard Pascal so that it could be readily transported to other computer systems. The program was then transferred to an IBM-PC system, and the display functions were added. At this point the portability criteria was considered to be of less importance than

---

150 Shofstahl, J.H. and Hardy, J.K., *J. Chem. Ed.*, **65**, A204 (1988).

taking advantage of the capabilities of the IBM-PC specific graphics and mouse hardware.

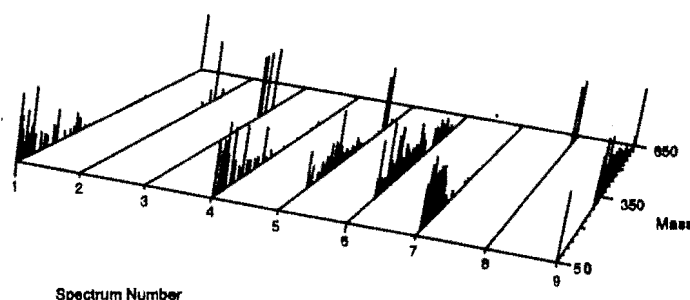
MassCalc functions fall into three categories. The first group of functions are calculations. The Calculate Mass (CM) function calculates the mean and lowest isotope molecular masses, and the weight-percentage of each element. Empirical or molecular formulae may be calculated from elemental analysis results using the Elemental Analysis (EA) function. The Isotope Pattern (IP) function generates the expected pattern of peaks for a molecular formula. The effect of overlapping M and M+1 peaks, of varying ratios, may be simulated. The example below shows the isotopic pattern plot for  $C_9H_9Br_3O_3$ , with an M+1 pattern, of half the M intensity, overlaid.



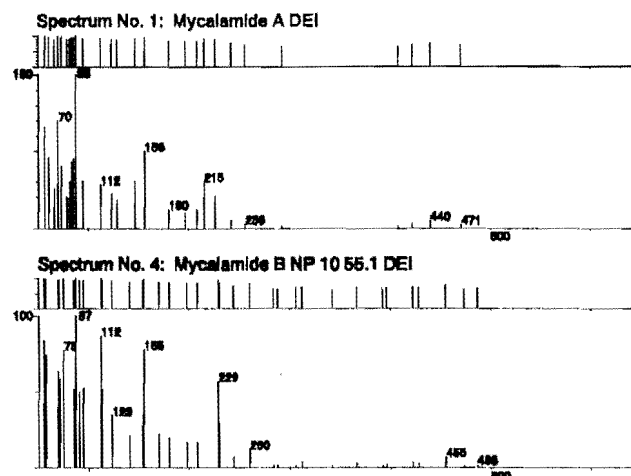
The second group of MassCalc functions are used for the display and analysis of stored spectra. The Print Parameters subfunction PP P lists the peaks for a particular spectrum. A table of differences may be generated for each pair of peaks with the Print Differences command (PD). The spectra in a file may be displayed in various ways using the Plot (PL) group of commands. If the spectra in a file being analysed are from a single chromatographic run, the sum of the peak intensities of each spectrum may be plotted against spectrum number with the PL TI command. Alternatively a series of spectra may be displayed as a three-dimensional projection with the PL 3D command. The plot in the example below shows each of the spectra in the spectrum file, normalised such that the base peak of each

spectrum has the same height. The data may also be normalised relative to the strongest peak in all the spectra being displayed, which would be more suitable for a spectrum file containing a GCMS run.

File: mycamd.MSD



Individual spectra may be displayed, or compared with another spectrum with the PL SP command. When two spectra are compared, the peaks which occur in both spectra are highlighted in colour, or with an asterisk on monochrome displays. Each spectrum is displayed twice, with the intensities of the upper scale being plotted on a four decade logarithmic scale to show the peaks of low intensity. The spectra of the compounds mycalamide A and B<sup>151</sup> are compared in the following example.



151 Perry, N.B., Blunt, J.W. and Pannell, L.K., *J. Am. Chem. Soc.*, **110**, 4850 (1988).

The second group of functions has three commands for analysing spectra. The Formula Search (FS) command generates possible molecular formulae for spectrum peaks or explicitly specified masses. The Find Formula (FF) command is effectively the inverse of the formula search. This command searches one or more of the spectra in the data file for occurrences of the specified formula. The masses and the sums and differences of each pair of masses in each of the spectra are compared with the target mass. The Find Formula function is particularly useful for searching for the presence of a known compound in the spectrum or spectra of a mixture. This function is also useful for elucidating the fragmentation of a compound. The Find Formula function was used in an attempt to interpret the high-resolution spectrum of tedanolide.<sup>152</sup> Plausible fragments were identified, and MassCalc was used to scan the spectrum for these.

In the first of the two examples, presented below, the spectrum was searched for the proposed  $C_{17}H_{25}O_5$  fragment. A single ion corresponding to this mass was found.

```
Searching for formula C17 H25 O5
Search mass = 309.17018
Match error = Automatic Selection
Sp  PkNo  Mass  Int%  Op  PkNo  Mass  Int%  Result  ppm  mmu
7:   2  309.17083  6  . . . . . = 309.17083  -2  -0.7
```

The spectrum was then searched for the  $C_9H_{15}O_2$  fragment. No peak was found for this mass, but it was found as a loss from peak 2 to peak 19, and as the sum of two other pairs of peaks.

```
Searching for formula C9 H15 O2
Search mass = 155.10719
Match error = Automatic Selection
Sp  PkNo  Mass  Int%  Op  PkNo  Mass  Int%  Result  ppm  mmu
7:   19  154.06324  47 minus   2  309.17083  6 = 155.10759  -3  -0.4
7:   120  73.06564  47 plus   111  82.04150  31 = 155.10714  0  0.1
7:   121  73.03030  48 plus   110  82.07671  60 = 155.10701  .1  0.2
```

The Automatic Search (AS) is the third analysis function. There are two sub-types of search. The first type is an extension of the Formula Search command, which

---

<sup>152</sup> Schmitz, F.A., Personal communication

allows all of the peaks in all of the spectra in the data file to be Formula Searched. The second sub-type of Automatic Search searches the spectra in the file for known fragments or combinations of one or more of the fragments. The Automatic Search is somewhat of a "blunderbuss" approach, and needs careful selection of the controlling parameters to yield useful results.

The third group of MassCalc commands are the "housekeeping" functions which display and change the setting of the parameters which control the program.

The atomic masses used by MassCalc are read from a disk file. The entries in this file may be elements or common fragments. The fragment entries are divided into two groups, called the fragment list and the alternate list. Typically the fragment list would contain generally applicable data, and the alternate list would have fragment masses that were applicable to a particular problem. Any entry in the file may be used in a molecular formula, but only items from the two fragment lists may be used in the second type of Automatic Search, described above.

The data which is displayed or analysed by the various functions may be restricted in three ways. The Intensity Cutoff (IC) parameter sets a lower limit of relative intensity. The Mass Range (MR) confines processing to peaks whose mass lies within the specified range, and the spectra which are used are determined by the Spectrum Scan Range (SR) parameter.

The comparison of masses in the program is controlled by the Match Error (ME) parameter. A matching tolerance may be explicitly set, or the appropriate limit may be automatically selected by the program. In the automatic mode the Match Error is set according to the precision to which the masses of the two entities being compared is specified. When either or both of the masses in the comparison have two or less digits after the decimal point, they are judged to be equal if they are less than half a mass unit apart. If both masses are more precise the Match Error is set to 2mmu.

The spectrum data file may contain a series of spectra from running a single sample, or a collection of reference spectra obtained from various sources. It is a



text file of mass/intensity pairs which may be created and edited with any text editor. The file may contain up to 1000 spectra, and each spectrum may have 5000 mass/intensity pairs. An IBM-PC with 640kb of memory is sufficient for 35,000 peaks. The file (MYCAMD.MSD) used in the above examples may be found on the diskette at the back of this volume. As may be seen in the sample below, the spectra in the file are delimited by *!BeginSpectrum* and *!EndSpectrum* statements and the file is terminated with an *!EndRun* statement. The mass/intensity pairs are entered in free-format.

```
!BeginSpectrum Mycalamide A DEI
  503 0.1 484 0.1 471 2.0 459 0.5 454 0.3 440 4.5 434 0.4 422 2.5
  414 1.0 408 1.5 392 0.5 360 1 346 1 328 1 312 1 292 1.5 274 1
!EndSpectrum

!BeginSpectrum Tedanolide EI!HiRes, ex F.J. Schmitz
  678.98101 193 309.17083 141 291.16005 129 221.15540 267 193.08820 186
  185.09058 351 175.06028 133 170.07384 355 169.08665 1087 168.05680 93
  167.07084 42 165.12851 228 165.09346 53 163.11288 59 163.07808 57
!EndSpectrum
!EndRun
```

The results from the various functions may be printed, or saved in a disk file. The graphical output is saved as Hewlett-Packard HPGL plotter commands.

MassCalc and the data files are on the diskette at the back of this thesis. The files MASSCALC.EXE, MASSCALC.ENV, MASSCALC.DAT and the example spectrum file MYCAMD.MSD should be copied from this diskette to a blank diskette or to an empty directory on a hard disk. Appendix A contains the complete user manual.

## **General Experimental**

---

The experimental work was carried out using the following procedures, equipment and materials.

### **Equipment and Materials**

Analytical and semi-preparative scale high pressure liquid chromatography was conducted using either a Varian 5020 Liquid Chromatograph with a Varian UV-50 UV/visible detector, or a Shimadzu LC-4A chromatograph with an SPD-2AS UV detector. Both chromatographs were equipped with Rheodyne 7125 injectors. The chromatograms were recorded using Hewlett-Packard 3390A integrators. Alltech and Dupont Zorbax micro-particle columns were used for analytical (4.5mm x 250mm columns) and semi-preparative (10mm x 250mm columns) HPLC

The preparative scale HPLC was carried out various configurations of the following equipment: An LKB 2132 Microperpex peristaltic pump, Rheodyne type 50 injectors, an LKB 2142 Differential Refractometer, LKB 2238 Uvicord II UV detectors, LKB 2212 Helirac fraction collectors, and Milton-Royal pumps. The preparative cyanopropyl and octyl columns used were Lobar (Merck LiChroprep). Davisil (20-30 $\mu$ ) was used to pack silica gel columns, and octadecyl columns were packed with bonded phase material prepared from the same grade of Davisil. Fractogel PGM2000 (Merck) was used for gel permeation chromatography. All tubing and fittings used were either stainless steel or Teflon.

Reverse phase flash chromatography was carried out in glass columns with packings prepared, as described in the next section, from 35-70 $\mu$  silica gel (Davisil). Silica gel (Grace 923) and Florisil (BDH) were used for adsorption mode chromatography.

Methanol for analytical and semi-preparative HPLC was chromatographic grade (J.T. Baker or Riedel-deHaën). Acetonitrile (BDH HiPerSolv) was used for reverse-phase chromatography to allow detection at 190nm. Water was purified using a MilliQ deionising system. All other solvents used were technical grade,

and were carefully purified, redistilled and dried prior to use.<sup>153</sup> The solvents used for HPLC were filtered prior to use (Millipore, 0.45 $\mu$ ). Pet. ether refers to light petroleum (boiling range 50-70°C), and ether to diethyl ether.

A Varian 3770 capillary gas chromatograph with a flame ionisation detector (FID) was used for gas chromatography, with helium was used as the carrier gas.

Infrared spectra were recorded using either a Pye Unicam SP3-300 Infrared Spectrophotometer, or a Shimadzu IR27G spectrophotometer. Ultraviolet spectra were obtained using a Varian Super Scan 3 Ultraviolet-visible spectrophotometer. Melting points were obtained using a Reichert 'Kofler' hot stage microscope and are uncorrected. The NMR spectra were recorded using a Varian XL300.

---

153. Riddick, J.A. and Toops, E.E., *Organic Solvents: Physical Properties and Methods of Purification*, 2nd Edn (Wiley Interscience: New York 1955).

## **Bioactive Compounds - Initial Studies**

---

### **Reverse-phase Test Column**

C-18 material (100g, 35-70 $\mu$ ) was slurried with methanol and packed into a flash column (27mm x 230mm), then equilibrated with water. Finely ground L-tyrosine, 1,3,5-trihydroxybenzene, phenylthiazole and cholesterol (approximately 200mg of each) were mixed and the mass was made up to 11g with sodium chloride. The test sample was dissolved in a mixture of methanol, water and dichloromethane and then slurried with C-18 material (10g). The solvents were removed under reduced pressure to yield a powder which was applied to the column.

A steep, stepped gradient from water to dichloromethane was used to elute fourteen fractions (H<sub>2</sub>O, 200ml, 9.9g; 30% CH<sub>3</sub>OH/H<sub>2</sub>O, 75ml, 98mg; 30% CH<sub>3</sub>OH/H<sub>2</sub>O, 75ml, 116mg; 50% CH<sub>3</sub>OH/H<sub>2</sub>O, 75ml, 105mg; 50% CH<sub>3</sub>OH/H<sub>2</sub>O, 75ml, 8.7mg; 65% CH<sub>3</sub>OH/H<sub>2</sub>O, 75ml, 8.3mg; 65% CH<sub>3</sub>OH/H<sub>2</sub>O, 75ml, 165mg; 80% CH<sub>3</sub>OH/H<sub>2</sub>O, 75ml, 82mg; 80% CH<sub>3</sub>OH/H<sub>2</sub>O, 75ml, 2.3mg; 95% CH<sub>3</sub>OH/H<sub>2</sub>O, 75ml, 2.8mg; 95% CH<sub>3</sub>OH/H<sub>2</sub>O, 75ml, 4.8mg; CH<sub>3</sub>OH 200ml, 31mg; 50% CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH 200ml, 215mg; CH<sub>2</sub>Cl<sub>2</sub> 200ml, 0mg). The composition of the first eleven fractions was measured by HPLC (C-18 Econosphere, 4.5mm x 250mm; 50% and 65% CH<sub>3</sub>OH/H<sub>2</sub>O at 1.5ml/min; UV 215nm). The final three fractions were shown to contain almost pure cholesterol by <sup>1</sup>H-NMR. The compositions were as follows. L-tyrosine, F2 90mg and F3 87mg, 1,3,5-trihydroxybenzene, F2 8mg, F3 30mg, F4 100mg, F5 8.7mg, F6 8.3mg and F7 15mg. phenylthiazole, F7 153mg, F8 82mg, F9 2.3mg and F10 2.8mg. cholesterol, F11 4.8mg, F12 31mg and F13 215mg. The compositions are accurate to within 15%.

### **Preparation of Reverse Phase Materials.**

The octadecyl reverse phase packings used for preparative and flash chromatography columns were prepared from silica gel (Davisil, 20-30 $\mu$  for preparative chromatography and 35-70 $\mu$  for MPLC) by the method similar to that of Kuhler and Lidsten,<sup>154</sup> and of Evans *et al*<sup>155</sup> using a 5% solution of octadecyltrichlorosilane (EGA-Chemie) in carbon tetrachloride. The silica gel was allowed to react for five minutes before washing with carbon tetrachloride (x2). The unreacted chloride groups were removed by washing with dry methanol. A dichloromethane solution of 5% trimethylchlorosilane (Aldrich) was used for "end-capping." The reverse-phase material was finally washed with dichloromethane (x2) then methanol (x2) to remove all traces of the reagents. The volume of solvents and reagents used was 10ml per gram of silica gel.

Bonded phase TLC plates were prepared from plastic backed silica gel TLC sheets (Merck Art 5735). The plates were reacted with octadecyltrichlorosilane (100ml, 5% w/v in CH<sub>2</sub>Cl<sub>2</sub>) or 3-cyanopropyl-dimethyl-chloro silane for 10 minutes in a flat tray. The reagent was removed by washing with dichloromethane (3 x 100ml), then dried at 40°C. The plates were "end-capped" with trimethylchlorosilane (100ml, 5% w/v in CH<sub>2</sub>Cl<sub>2</sub>) and washed to remove excess reagent. Great care was needed to prevent the separation of the silica gel from the plastic backing during the latter stages of the process.

### ***Aplidium gilvum***

Fresh *A. gilvum* (4K4-4, 2.1kg) was homogenised in 75% methanol/toluene (800ml), filtered and the residue repeatedly resuspended (2 x 400ml 75% CH<sub>3</sub>OH/toluene then 1 x 400ml 50% CH<sub>3</sub>OH/H<sub>2</sub>O). Methanol (1l) was added to the combined extracts until it was a single phase. The mass of the extract (20g) was determined by removing the solvent from an aliquot.

---

154. Kuhler, T.C. and Lidsten, G., *J. Org. Chem.*, **48**, 3589 (1983).

155. Evans, M.B., Dale, A.D. and Little, C.J., *Chromatographia*, **13**, 1, 5 (1980).

The solvents were removed, *in vacuo*, from an aliquot of the extract (100ml). A 90% methanol/water solution/suspension (100ml) of the extract was made by washing the residue first with methanol, then with water, and combining the solutions. The sample was extracted with pet. ether (3 x 50ml). Water was added to bring the methanol/water fraction to 70% methanol, before it was extracted with dichloromethane (3 x 50ml). More water was added until the composition was 30% methanol. The methanol/water fraction was extracted with ethyl acetate (3 x 50ml). Finally the methanol was removed, under reduced pressure, and the remaining aqueous layer was extracted with butan-1-ol.

The volume of each fraction was adjusted to 100ml for the antiviral assay (pet. ether,  $\pm$ ,  $-$ ,  $-$  40 $\mu$ g; CH<sub>2</sub>Cl<sub>2</sub>  $-$ ,  $-$ ,  $-$  40 $\mu$ g; EtOAc  $-$ ,  $-$ ,  $-$  40 $\mu$ g; nBuOH ++, ++, ++ 40 $\mu$ g; H<sub>2</sub>O +, +,  $\pm$  40 $\mu$ g).

An aliquot of the butan-1-ol fraction was applied as a band to a deactivated silica gel TLC plate and eluted with 50% methanol/dichloromethane. The plate was divided into three bands at R<sub>f</sub> 0.11 and 0.49. The absorbed compounds were recovered from the scrapings by washing with methanol, and submitted for antiviral assay, along with a reagent blank (R<sub>f</sub> 0.0-0.11  $-$ ,  $-$ ,  $-$  ?; 0.11-0.49  $-$ ,  $-$ ,  $-$  ?; 0.49-1.0 +++, ?, +++ ?; blank  $-$ ,  $-$ ,  $-$  ?).

A further aliquot (880mg) of the crude extract was dissolved/suspended in methanol, then coated onto Florisil (BDH). The sample was then loaded onto a Florisil column (10mm x 140mm). Twenty-six fractions (10ml) were eluted (F1, F2 pet ether; F3, F4 0.5% Et<sub>2</sub>O/pet. ether; F5, F6 1% Et<sub>2</sub>O; F7, F8 5% Et<sub>2</sub>O; F9, F10 10% Et<sub>2</sub>O; F11, F12 50% Et<sub>2</sub>O; F13, F14 Et<sub>2</sub>O; F15-F17 50% EtOAc/Et<sub>2</sub>O; F18, F19 EtOAc; F20, F21 5% CH<sub>3</sub>OH/EtOAc; F22, F23 20% CH<sub>3</sub>OH; F24, F25 50% CH<sub>3</sub>OH; CH<sub>3</sub>OH 50ml). The fractions were combined after analysis by silica gel TLC, eluting with ether, and submitted for antiviral assay (F1-F6 0.7mg,  $-$ ,  $-$ ,  $-$  40 $\mu$ g; F7-F10 0.4mg,  $-$ ,  $-$ ,  $-$  40 $\mu$ g; F11-F13 3.8mg,  $-$ ,  $-$ , + 40 $\mu$ g; F14-F18 2.1mg,  $-$ ,  $-$ ,  $-$  40 $\mu$ g; F19-F24 16.2mg +++, ++,  $\pm$  40 $\mu$ g; F25 130mg, ?, ?, +++ 10 $\mu$ g).

The organic solvents were removed from the remainder of the extract, under reduced pressure, to yield a yellow aqueous solution/suspension (0.75l), which

was extracted with ethyl acetate (1 x 200ml). Further attempts to extract with ethyl acetate resulted in emulsions which could not be broken. The aqueous layer was then extracted with butan-1-ol (3 x 200ml). The antiviral assay revealed activity in the ethyl acetate and aqueous fractions (ethyl acetate  $?, ?, ++$  40 $\mu$ g; aqueous  $?, ?, +++$  20 $\mu$ g and  $-, -, -$  5 $\mu$ g). The three fractions were combined.

One half of the crude extract was coated onto C-8 (Lichrosorb) material (8g) by removing the solvents from a methanol/water slurry. The dried powder was added to a reverse-phase flash column (90g C-8, 20mm x 200mm), and eluted with a steep, stepped gradient. Forty-four fractions (25ml) were collected (F1-F4 H<sub>2</sub>O; F5-F8 5% CH<sub>3</sub>OH/H<sub>2</sub>O; F9-F16 50% CH<sub>3</sub>OH/H<sub>2</sub>O; F17-F20 80% CH<sub>3</sub>OH/H<sub>2</sub>O; F21-F24 95% CH<sub>3</sub>OH/H<sub>2</sub>O; F25-F32 CH<sub>3</sub>OH; F33-F36 5% CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH; F37-F40 20%CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH; F41-F44 CH<sub>2</sub>Cl<sub>2</sub>; strip, 200ml CH<sub>2</sub>Cl<sub>2</sub> and 300ml 50% CH<sub>2</sub>Cl<sub>2</sub>/pet. ether). These fractions were combined into six on the basis of TLC (Whatman C-18 plates, developed with 80% CH<sub>3</sub>OH/H<sub>2</sub>O or 50% CH<sub>3</sub>OH/1M aqueous NaCl) and submitted for antiviral assays (F1-F8 7g,  $-, -, -$  40 $\mu$ g; F9-F16 210mg,  $-, -, -$  40 $\mu$ g; F17-F20 32mg,  $-, -, -$  40 $\mu$ g; F21-F25 143mg,  $?, ?, +++$  40 $\mu$ g; F26-F30 250mg,  $?, ?, ++$  40 $\mu$ g; F30-strip 190mg,  $?, ?, \pm$  40 $\mu$ g).

### ***Margaretta barbata***

*M. barbata* (2.1kg, voucher 831130-02) was homogenised with 50% propan-2-ol/dichloromethane (2.5l) and filtered. The residue was resuspended once in 50% propan-2-ol/dichloromethane (750ml) and twice in 50% propan-2-ol/water (750ml). Removal of the solvents, *in vacuo*, yielded a dark brown tar (50g).

An aliquot of the extract (1.5g) was dissolved/suspended in 70% methanol/water (60ml) and extracted with dichloromethane (4 x 75ml). The methanol was removed from the aqueous fraction, under reduced pressure, which was then extracted with ethyl acetate (3 x 50ml). The aqueous layer was finally extracted with butan-1-ol (4 x 75ml). The butan-1-ol fraction was dissolved in water (50ml) and re-extracted with butan-1-ol (3 x 20ml). The four fractions were submitted for antiviral assay

(CH<sub>2</sub>Cl<sub>2</sub> 68mg, --- 40µg; EtOAc 20mg, --- 40µg; nBuOH 17.2mg --- 40µg; aqueous 1.3g, ±,±,- 40µg and +,?,+++ 160µg).

Another aliquot of the crude extract (1.2g, perhaps 150mg of organic soluble material) was chromatographed on Florisil (BDH). The extract was coated onto Florisil, by evaporation, *in vacuo*, of a methanol solution, and added to a column (10mm x 150mm). Twenty-six fractions (12ml, except for the methanol strip which was 50ml) were collected. Two fractions of each solvent compositions were collected (pet. ether, 0.5% Et<sub>2</sub>O/pet. ether, 1% Et<sub>2</sub>O/pet. ether, 5% Et<sub>2</sub>O/pet. ether, 10% Et<sub>2</sub>O/pet. ether, 50% Et<sub>2</sub>O/pet. ether, Et<sub>2</sub>O, 50% Et<sub>2</sub>O/EtOAc, EtOAc, 10% propan-2-ol/EtOAc, 50% propan-2-ol, propan-2-ol, methanol). The fractions were combined into eight fractions, after TLC analysis (silica gel, developed with ether, visualised with phosphomolybdic acid), for antiviral assay (F1-F10 4.0mg, +,+,+,- 40µg; F11-F12 14mg, +,-,- 40µg; F13-F14 4.4mg, +,±,- 40µg; F15-F17 3.3mg, -,+,? 40µg; F18-F19 8.0mg, --- 40µg; F20-F22 1.3mg, +,-,- 40µg; F23-F25 2.7mg, +,-,- 40µg; F26 (strip) 93mg, ?,?,+++ 40µg).

The first fraction from the previous step was chromatographed by normal-phase HPLC (CN Zorbax 10mm x 250mm; 2% propan-2-ol/hexane, 5ml/min; UV 210nm). Three fractions and a 20% propan-2-ol wash were collected (2.9mg, 0.2mg, 0.4mg, 0.3mg). No antiviral or cytotoxic activity was detected in any of the fractions.

A further portion of the crude extract (3g) was partitioned between water and dichloromethane (200ml H<sub>2</sub>O, extracted with 3 x 200ml CH<sub>2</sub>Cl<sub>2</sub>; antiviral assays: H<sub>2</sub>O ±,--- 40µg; CH<sub>2</sub>Cl<sub>2</sub> --- 40µg). The aqueous soluble material was then freeze dried (2.5g). The residue was washed with dry methanol (4 x 20ml, 1.14g) to remove the organic material, then with water (3 x 10ml, 1.4g). The residue was 22mg.

The methanol soluble material was chromatographed using on a reverse-phase flash column (30g C-18, 28mm x 150mm). The sample (1g) was coated onto a quantity of C-8 (3g) from a methanol solution. Thirty seven fractions (25ml) were eluted with a stepped gradient from 50% methanol/water, through methanol to



80% dichloromethane/methanol (F1-F6 50% CH<sub>3</sub>OH/H<sub>2</sub>O; F7-F12 80% CH<sub>3</sub>OH/H<sub>2</sub>O; F13-F16 95% CH<sub>3</sub>OH/H<sub>2</sub>O; F17-F22 CH<sub>3</sub>OH; F23-F26 1% CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH; F27-F30 5% CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH; F31-F37 80% CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH; strip 200ml CH<sub>2</sub>Cl<sub>2</sub> and 1l 20% pet. ether/CH<sub>2</sub>Cl<sub>2</sub>). After analysis by reverse-phase HPLC (C-18 Zorbax 4.5mm x 250mm; 80% CH<sub>3</sub>OH/H<sub>2</sub>O or CH<sub>3</sub>OH, 1.5ml/min; UV 210nm), the column fractions were combined into six fraction for antiviral assay (F1-F4 852mg, ---, 40µg; F5-F9 28mg, ---, 40µg; F10-F15 9.4mg, ---, + 40µg; F16-F18 20mg, ?,?,++ 40µg; F19-F21 14mg, ---, + 40µg; F22-strip 42.1mg ---, 40µg).

Another collection of *M. barbata* (850g, voucher number 4K4-2), carefully cleaned of all epibionts, was homogenised with 75% methanol/toluene (750ml). The residue was re-extracted with 75% methanol/toulene (3 x 400ml) and 50% methanol/water (2 x 400ml). The extract weight (25g) was determined by evaporating an aliquot of the solution.

One half of the extract (12.8g) was coated onto an equal weight of C-8 material, then applied to a C-8 column (20mm x 200mm). Forty-eight fractions (25ml) were collected before the column was stripped (F1-F4 H<sub>2</sub>O; F5-F8 5% CH<sub>3</sub>OH/H<sub>2</sub>O; F8-F12 50% CH<sub>3</sub>OH/H<sub>2</sub>O; F12-F16 60% CH<sub>3</sub>OH/H<sub>2</sub>O; F17-F20 90% CH<sub>3</sub>OH/H<sub>2</sub>O; F21-F28 95% CH<sub>3</sub>OH/H<sub>2</sub>O; F29-F36 100% CH<sub>3</sub>OH/H<sub>2</sub>O; F37-F40 2% CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH; F41-F44 20%CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH; F44-F48 CH<sub>2</sub>Cl<sub>2</sub>; stripped with 1l CH<sub>2</sub>Cl<sub>2</sub>, 200ml 20% pet. ether/CH<sub>2</sub>Cl<sub>2</sub>). The fractions were analysed by reverse-phase TLC (Whatman C-18, developed with 50% CH<sub>3</sub>OH/1M aqueous NaCl, 70% CH<sub>3</sub>OH/H<sub>2</sub>O, 95% CH<sub>3</sub>OH/H<sub>2</sub>O, 5% CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH or 20% CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH, visualised with phosphomolybdic acid), and combined into seven fractions for assay (F1-F4 9.1g, ---,++ 40µg; F5-F11 1.4g, ---,+++ 40µg; F12-F16 159mg, ---, 40µg; F17-F23 67mg, ?,?,+++ 5µg; F24-F31 179mg, ---,++ 40µg; F32-F41 366mg, ---, 40µg; F42-strip 272, ---, + 40µg).

The combined fractions F17-F23 (50mg) from the previous step were applied to a C-8 Lobar MPLC column. Five fractions (100ml) were eluted with a stepped gradient (F1 13.7mg, 70% CH<sub>3</sub>OH/H<sub>2</sub>O, ---,++ 10µg; F2 8.6mg, 75% CH<sub>3</sub>OH/H<sub>2</sub>O, ---,++ 10µg; F3 10mg, 85% CH<sub>3</sub>OH/H<sub>2</sub>O, ---,++ 10µg; F4 4.6mg, 95%

CH<sub>3</sub>OH/H<sub>2</sub>O, --,+++ 10μg; strip 2.5mg, CH<sub>3</sub>OH, 50% CH<sub>3</sub>OH/CH<sub>2</sub>Cl<sub>2</sub>, --,--  
10μg).

## **Compounds from *Tedania* Species**

---

### **Extraction of PML1-6: Extract A**

A sample of *Tedania* n. sp. B (300g wet weight, collection number PML1-6) was homogenised in methanol/toluene (3:1, 500ml). The homogenate was filtered and resuspended/filtered twice more in the same methanol/toluene mixture (2 x 500ml). The solvents were removed *in vacuo* to yield a brown tar (sample T1, 37g).

An aliquot of T1 (10g) was chromatographed by reverse-phase flash chromatography on Lobar C-8 support (25mm x 230mm column). A solution/suspension of the sample was slurried with C-8 support and then taken down to dryness under reduced pressure. Twelve 100ml fractions were eluted with a stepped solvent gradient from water through to methanol and dichloromethane (H<sub>2</sub>O, 2.77g; 10% CH<sub>3</sub>OH, 1.05g; 35% CH<sub>3</sub>OH/H<sub>2</sub>O, 116mg; 50% CH<sub>3</sub>OH/H<sub>2</sub>O, 52mg; 70% CH<sub>3</sub>OH/H<sub>2</sub>O, 51mg; 80% CH<sub>3</sub>OH/H<sub>2</sub>O, 48mg; 90% CH<sub>3</sub>OH/H<sub>2</sub>O, 14mg; CH<sub>3</sub>OH, 35mg; 10% CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH, 94mg; 10% CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH, 150mg; 50% CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH, 45mg; CH<sub>2</sub>Cl<sub>2</sub>, 232mg). The antiviral material was contained mainly in fractions seven and eight (F1 to F4 --,-- 40µg; F5 --,± 4µg; F6 ?,?,++ 5µg; F7 and F8 ?,?,+++ 5µg; F9 and F10 --,+, 5µg; F11 --,++, 5µg; F12 --,+++ 40µg).

Another ten gram aliquot of T1 was similarly chromatographed. (H<sub>2</sub>O, 6.54g; 10% CH<sub>3</sub>OH, 308mg; 35% CH<sub>3</sub>OH/H<sub>2</sub>O, 135mg; 50% CH<sub>3</sub>OH/H<sub>2</sub>O, 118mg; 70% CH<sub>3</sub>OH/H<sub>2</sub>O, 108mg; 80% CH<sub>3</sub>OH/H<sub>2</sub>O, 54mg; 90% CH<sub>3</sub>OH/H<sub>2</sub>O, 21mg; 95% CH<sub>3</sub>OH/H<sub>2</sub>O, 65mg; CH<sub>3</sub>OH, 68mg; CH<sub>3</sub>OH, 219mg; 10% CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH, 155mg; 50% CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH, 459mg). The antiviral material was contained mainly in fractions six and seven (F1 to F2 --,-- 40µg; F3 to F5 --,+, 2µg; F6 ++,+++, 40µg; F7 ?,?,+++ 40µg; F8 --,+, 40µg; F9 to F12 --,-- 40µg).

The remainder of T1 was chromatographed in the same system. (H<sub>2</sub>O, 4.19g; 10% CH<sub>3</sub>OH, 636mg; 35% CH<sub>3</sub>OH/H<sub>2</sub>O, 123mg; 50% CH<sub>3</sub>OH/H<sub>2</sub>O, 89mg; 70% CH<sub>3</sub>OH/H<sub>2</sub>O, 24mg; 80% CH<sub>3</sub>OH/H<sub>2</sub>O, 25mg; 90% CH<sub>3</sub>OH/H<sub>2</sub>O, 16mg; 95%

CH<sub>3</sub>OH/H<sub>2</sub>O, 35mg, CH<sub>3</sub>OH, 56mg; CH<sub>3</sub>OH, 137mg; strip with 50% CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH then CH<sub>3</sub>OH 231mg). The antiviral material was contained mainly in fractions six and seven (F1 to F5 --,-- 40µg; F6 +++,?,+++ 4µg; F7 ++,+++,+ 4µg; F8 --,+,+ 4µg; F9 to F11 --,-- 40µg).

The active fractions listed for the previous columns were combined as sample T2 (192mg). The entire amount of this sample was dissolved in methanol and coated onto C-8 support (10g) by removing the solvent under reduced pressure. The coated support was packed into a pre-column (35mm x 28mm dia.) which was connected to an MPLC column (Lobar). Twenty five fractions were eluted using a stepped gradient. The fractions were 50ml, except for fraction one (150ml) and fraction ten to twenty four (25ml). Fraction twenty-five was a methanol strip (300ml). (50% CH<sub>3</sub>OH/H<sub>2</sub>O, 26mg; 60% CH<sub>3</sub>OH/H<sub>2</sub>O, 10mg; 60% CH<sub>3</sub>OH/H<sub>2</sub>O, 5.3mg; 70% CH<sub>3</sub>OH/H<sub>2</sub>O, 5.7mg; 70% CH<sub>3</sub>OH/H<sub>2</sub>O, 7.2mg; 70% CH<sub>3</sub>OH/H<sub>2</sub>O, 6.6mg; 70% CH<sub>3</sub>OH/H<sub>2</sub>O, 6.1mg; 80% CH<sub>3</sub>OH/H<sub>2</sub>O, 9.8mg; 80% CH<sub>3</sub>OH/H<sub>2</sub>O, 14mg; 80% CH<sub>3</sub>OH/H<sub>2</sub>O, 19mg; 80% CH<sub>3</sub>OH/H<sub>2</sub>O, 11mg; 80% CH<sub>3</sub>OH/H<sub>2</sub>O, 8.4mg; 80% CH<sub>3</sub>OH/H<sub>2</sub>O, 7.9mg; F14, F15 90% CH<sub>3</sub>OH/H<sub>2</sub>O, 10mg; F16, F17 90% CH<sub>3</sub>OH/H<sub>2</sub>O, 6.8mg; F18, F19 90% CH<sub>3</sub>OH/H<sub>2</sub>O, 13.5mg; F20, F21 90% CH<sub>3</sub>OH/H<sub>2</sub>O, 17.9mg; F22 to F25 95%CH<sub>3</sub>OH/H<sub>2</sub>O, 19.5mg). The most active fractions were ten through twelve which were combined as sample T3 (30mg). (F1 to F6 --,+,+ 10µg; F7 --,++ 10µg; F8, F9 --,-- 10µg; F10 WW,?,++ 1µg; F11 WW,WW,- 1µg; F12 ?,?,+++ 10µg; F13, F14 --,++,+ 10µg; F16 --,+++,+ 10µg; F18 --,++,+ 10µg; F20, F22 --,+,+ 10µg; F25 --,--,10µg).

A sample of T3 was applied as a band to a reverse-phase (Whatman C-18) TLC plate. The plate was eluted with 70% methanol water. Visualising the edge of the plate with phosphomolybdic acid revealed spots at R<sub>f</sub> 0.15, 0.23, 0.33, 0.37, 0.49, 0.60, 0.74. The plate was cut in half, longitudinally. One half was placed adsorbent side down in a 40mm well infected with HSV. A 25mm semicircle of the cell sheet around the centre of the plate was free of CPE. This was not sufficiently localised to be of use.

The other half of the plate was divided into six bands (the divisions were at  $R_f$  0.19, 0.28, 0.40, 0.53, 0.65). The scrapings were washed with methanol and the washings assayed. Fractions two to four were strongly active (F1 ++,nd,+; F2 to F4 ?,nd,WW; F5,F6 and blank -,nd,+).

The remainder of T3 was chromatographed, in five aliquots, by reverse-phase HPLC (C-18 Alltech 10mm x 250mm, 70% CH<sub>3</sub>OH/H<sub>2</sub>O at 5ml/min, UV at 210nm). Twenty-five fractions (2.5ml) were taken from the poorly resolved envelope. The column effluent was assayed directly (F1 to F6 -,,-,?; F7, F8 -,,-,±?; F9 ?,?,+++?; F10 ? ++,++?; F11 +++,?,++?; F12 +++,++++,++?; F13 ++,++++,++?; F14 to F25 -,,-,?).

### **Extraction of 5P2-24: Extract B**

---

A 1kg sample of *Tedania connectens* Brøndsted, collection number 5P2-24, was homogenised using an Ultraturrax in methanol (1l), and the homogenate filtered (Whatman No.1). The residue was resuspended and filtered several more times (2 x 1 70% CH<sub>3</sub>OH/H<sub>2</sub>O, 1 x 1l 100% CH<sub>3</sub>OH and 3 x 0.75l CH<sub>2</sub>Cl<sub>2</sub>), with the dichloromethane extract being kept separate. Assay samples of these two extracts were made up to the equivalent of the screening concentration (2g sponge/20ml solvent). The antiviral assays indicated that all the active material had been removed by the water/methanol extraction (++++,++ for the main extract). The solvent of the active extract was reduced *in vacuo* (below 30°C) until no organic solvent remained (450ml). The mass of the total solids (38g) of this extract, T4, was determined by complete evaporation of an aliquot.

Initial purification was by RP column chromatography. The aqueous suspension of the extract was slurried with RP-18 (50g, 35-70µ) and poured into a RP-18 flash column (45mm x 210mm, 70g RP-18). Eight fractions were collected from the column which was eluted with a steep, stepped gradient from water through to methanol then stripped with methanol and dichloromethane (H<sub>2</sub>O, 600ml, 35.65g; 20% CH<sub>3</sub>OH/H<sub>2</sub>O, 300ml, 880mg; 50% CH<sub>3</sub>OH/H<sub>2</sub>O, 300ml, 588mg; 70% CH<sub>3</sub>OH/H<sub>2</sub>O, 300ml, 341 mg; 80% CH<sub>3</sub>OH/H<sub>2</sub>O, 300ml, 186mg; 90%

CH<sub>3</sub>OH/H<sub>2</sub>O, 300ml, 167mg; 95% CH<sub>3</sub>OH/H<sub>2</sub>O, 300ml, 264mg; CH<sub>3</sub>OH, 300ml, then 20% CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH, 300ml, then CH<sub>2</sub>Cl<sub>2</sub>, 300ml, 184mg). The antiviral activity was located in fractions five and six (F5 WW,WW,+ /C4 at 0.5µg; F6 -,+++,+ /C4/C7 at 20µg; fractions 2, 3, 7 showed slight cytotoxicity only at 20µg).

Analysis of fractions four and five by TLC (Whatman C-18, 70% CH<sub>3</sub>OH/H<sub>2</sub>O, visualised by phosphomolybdic acid) indicated at least six major compounds in each fraction. Several of the spots were ninhydrin positive.

The active fractions were combined in methanol/water and organic solvent was removed under reduced pressure. The solution/suspension (300mg in 10ml) was added to wetted RP-18 (2g), packed as a slurry on an RP-18 column (27mm x 230mm, 120g of 35-70µ), and eluted with a methanol/water gradient collecting fifteen fractions (50% CH<sub>3</sub>OH/H<sub>2</sub>O, 125ml, 9mg; 60% CH<sub>3</sub>OH, 75ml, 5mg; 60% CH<sub>3</sub>OH, 75ml, 7mg; fractions 4 to 7 were 70% CH<sub>3</sub>OH, 75ml each, 17mg, 52mg, 28mg and 20mg respectively; fractions 8 and 9 75% CH<sub>3</sub>OH, 75ml each, 10mg, 11mg; fraction 10 and 11 80% CH<sub>3</sub>OH, 75ml each, 10mg, 12mg; fractions 12 and 13 85% CH<sub>3</sub>OH, 75ml each, 8mg, 9mg; 90% CH<sub>3</sub>OH, 150ml, 17mg; stripped with 100% CH<sub>3</sub>OH, 100ml then 20% CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH, 150ml, 26mg). The active material was contained in fractions five, six and seven (F5 WW,WW,++ /C7 at 2.5µg and ++,+++,++ /C7 at 0.25µg; F6 WW,WW,+++ /C7 at 2.5µg and ++,+++,++ /C7 at 0.25µg; F7 WW,WW,+++ /C7 at 2.5µg and ++,+++,++ /C7 at 0.25µg; fractions 8, 9 and 11 showed slight C7 cytotoxicity at 2.5µg). Investigation of the three active fractions and the starting material for this column indicated that they were complex mixtures (C-18 Alltech 4.5mm x 250mm, 80% CH<sub>3</sub>OH/H<sub>2</sub>O at 1.5ml/min, UV at 220nm).

The three active fractions 5, 6 and 7 were combined (106mg). Once the solvent had been removed from these fractions they could not be completely redissolved in methanol/water mixtures. The undissolved residue was filtered off and the remaining 88mg of the mixture was purified by HPLC (C-18 Alltech 10mm x 250mm, 65% CH<sub>3</sub>OH/H<sub>2</sub>O at 5ml/min, UV detection at 220nm). Thirteen fractions, including the methanol strip were collected. Fractions two to ten were

2.5ml, fraction one was 7.5ml of eluant and fraction twelve was 5ml. The following masses were recorded for the fractions: 0.2mg, 1.9mg, 10.7mg, 39.3mg, 5.9mg, 2.1mg, 1.9mg, 1.9mg, 0.7mg, 1.3mg, 3.2mg, 6.4mg, 10.8mg. The antiviral activity was contained in fractions seven, eight and nine (F7, +++,nd,- at 0.5µg, none at 0.05µg; F8, WW,nd,- at 0.05µg; F9, WW,nd,- at 0.5µg, none at 0.05µg). Analytical HPLC (C-18 Alltech 4.5mm x 250mm, 65% CH<sub>3</sub>OH/H<sub>2</sub>O, uv 220nm) of these fractions indicated that they were still complex mixtures.

Analytical scale HPLC (C-18 Alltech 4.5mm x 250mm, 65%CH<sub>3</sub>OH/H<sub>2</sub>O at 1.5ml/min, UV 220nm) was used to further purify the active material.

Approximately 10µg was injected and five fractions (of 4.5, 0.75, 0.6, 0.75 and 6ml respectively) were taken, guided by peaks in the uv chromatogram. The entire amount of each fraction was transferred to the assay disks. The antiviral activity was located in the final fraction.

### ***Extraction of 5P2-24 and 5P3-1: Extract C***

---

*Tedania connectens* was collected from the channel near the Portobello Marine Laboratory, in the Otago Harbour (collection numbers 5P3-1 and 5P2-24).

A trial extraction to check the efficiency of the proposed method was carried out. 1kg of wet sponge (5P2-24) was freeze dried and the dried sponge (133g) blended using an Ultraturrax in 80% CH<sub>3</sub>OH/H<sub>2</sub>O (500ml) and filtered (Whatman No.1). The residue was resuspended in fresh solvent (80% CH<sub>3</sub>OH/H<sub>2</sub>O, 500ml) and filtered, three more times. The residue was extracted twice more with methanol (500ml), and three times with dichloromethane (500ml). The methanol/aqueous, methanol and dichloromethane extracts were then assayed separately. (++,-,+/C7 -, -,+/C7 and -, -, - respectively).

5P3-1 (7.2kg of wet sponge) was homogenised (Waring blender) in 6l of methanol and filtered. The residue was extracted three times with 90% CH<sub>3</sub>OH/H<sub>2</sub>O (1 x 4l, 2 x 2l). One further extraction, with the same solvents, was done and kept separate for assay to check that all the active material had been removed. Antiviral assay: for the bulk extract, WW,WW,++/C7 and +++ for *Adenovirus* at 100µg; no activity

detected in the final extract). The solvents were removed from the extract under reduced pressure by cyclone evaporator, while the temperature was kept below 35.° The mass of the extract (259g) was measured by taking a 10ml aliquot of the extract to dryness.

The remainder of 5P3-1 (10kg) and 5P2-24 (7kg) were extracted by the same method (1 x 12l CH<sub>3</sub>OH, and 4 x 5l 90% CH<sub>3</sub>OH/H<sub>2</sub>O), and the solvent removed using a cyclone evaporator to yield 1kg of a brown malodorous tar (sample T8).

Antiviral assay: WW,nd,+ / C7 and + and WW for Echo6 and Adenovirus respectively.

### ***Trial Column of T8***

Fifteen grams of rather wet T8 was slurried with pre-wetted C-18 (15g, 35-70μ) and water (25ml), then packed onto a C-18 flash column (25mm x 200mm, 60g C-18, 35-70μ) and eluted with a steep stepped gradient of water/methanol mixtures. Seven fractions including the strip were collected (H<sub>2</sub>O, 200ml, 14.5g; 20% CH<sub>3</sub>OH/H<sub>2</sub>O, 150ml, 270mg; 50% CH<sub>3</sub>OH/H<sub>2</sub>O, 100ml, 143mg; 70% CH<sub>3</sub>OH/H<sub>2</sub>O, 100ml, 117mg; 80% CH<sub>3</sub>OH/H<sub>2</sub>O, 200ml, 59mg; 90% CH<sub>3</sub>OH/H<sub>2</sub>O, 100ml, 38mg; stripped with 100% CH<sub>3</sub>OH, 100ml, then CH<sub>2</sub>Cl<sub>2</sub>, 200ml then 50% CH<sub>2</sub>Cl<sub>2</sub>/pet. ether, 150ml, then methanol, 200ml, 195mg; ). The solvents were removed *in vacuo* and several drops of octan-1-ol were added to fraction five to prevent foaming. Fraction five was active in the antiviral assay (WW,WW,- at 5μg).

### ***Large Scale Purification of T8***

A portion of T8 (120g) was slurried with water (150ml) and C-18 (150g), then added to a short C-18 flash column (90mm dia. x 130mm, 300g C-18, 35-70μ). Seven fractions were collected, including the strip (H<sub>2</sub>O, 2.4l; 50% CH<sub>3</sub>OH/H<sub>2</sub>O, 1l; 50% CH<sub>3</sub>OH/H<sub>2</sub>O, 1l; 80% CH<sub>3</sub>OH/H<sub>2</sub>O, 2l; 80% CH<sub>3</sub>OH/H<sub>2</sub>O, 1l; 80% CH<sub>3</sub>OH/H<sub>2</sub>O, 1l; stripped with CH<sub>3</sub>OH, 2l then CH<sub>2</sub>Cl<sub>2</sub>, 2l then CH<sub>3</sub>OH, 1l). The



antiviral activity was contained completely in fraction four (WW,WW,++/C4/C7 at 40µl of column effluent per disk).

This separation procedure was repeated twice more, once with 120g of extract B then five times with 160g, using an optimised gradient (H<sub>2</sub>O, 2.5l; 50% CH<sub>3</sub>OH/H<sub>2</sub>O, 2l; 80% CH<sub>3</sub>OH/H<sub>2</sub>O, 4l; stripped with 1l CH<sub>3</sub>OH, then 500ml 25% CH<sub>3</sub>OH/CH<sub>2</sub>Cl<sub>2</sub>, then 1.5l CH<sub>3</sub>OH with 0.05% trifluoroacetic acid (TFA)).

In each of these seven separations the antiviral material was eluted by the 80% methanol/water mixture. The active fractions (12.8g) were combined (antiviral activity: +++,+,+,+ at 8µg) and the solvents removed under reduced pressure using a cyclone evaporator.

On standing at 4°C, in 90% CH<sub>3</sub>OH/H<sub>2</sub>O, the active fraction threw down a fine white precipitate (1.06g) which was filtered off. The filtrate was washed with methanol and the washings added to the active material. The precipitate was insoluble in water, methanol or dichloromethane, but was moderately soluble in 0.05% TFA/CH<sub>3</sub>OH. It exhibited no antiviral activity (at 10µg/disk) and had a relatively featureless NMR spectrum.

The active material was applied to a C-18 column (90mm x 150mm, 500g C-18, 35-70µ, carefully packed in CCl<sub>4</sub>) as a C-18/water slurry, and eluted using water/methanol (50% CH<sub>3</sub>OH/H<sub>2</sub>O, 2l, 2.49g; 65% CH<sub>3</sub>OH/H<sub>2</sub>O, 1l, 2.27g; 65% CH<sub>3</sub>OH/H<sub>2</sub>O, 1l, 1.06g; 80% CH<sub>3</sub>OH/H<sub>2</sub>O, 1l, 1.2g; 80% CH<sub>3</sub>OH/H<sub>2</sub>O, 1l, 1.31; 80% CH<sub>3</sub>OH/H<sub>2</sub>O, 1l, 1.41g; 80% CH<sub>3</sub>OH/H<sub>2</sub>O, 400ml, 0.36g; CH<sub>3</sub>OH, 2l, 2.3g; 0.05% TFA/CH<sub>3</sub>OH, 2l, 1.17g). The column eluant was assayed and the concentrations were calculated after the solvent had been removed. The activity was located in fractions two and three (F2 ?,?,+++/C7 at 35µg; F3 +++,+,+,+/C7 at 5µg). The remaining fractions exhibited some cytotoxicity, but no antiviral activity, at 40µg or more per disk.

### ***Further Purification of the Antiviral Material, by MPLC***

A steel MPLC column (20mm x 350mm) was packed with C-18 (Davisil, 20-30 $\mu$ ) slurry in carbon tetrachloride. The steel tube was extended with a glass tube so that the entire volume of slurry could be added at once. After settling (24 hours) the column was pumped with carbon tetrachloride, then opened and topped up. Finally the column was washed with methanol and equilibrated with 70% CH<sub>3</sub>OH/H<sub>2</sub>O.

A portion of the fraction two (200mg) from the previous step was applied to the MPLC column, and eluted with 70% CH<sub>3</sub>OH/H<sub>2</sub>O (7ml/min). The separation was followed by UV absorbance (205nm), and nineteen fractions were collected after the column dead-volume (75ml) had been discarded. The antiviral assay indicated a very poor separation, and the active fractions were combined and added to the starting material. The two active fractions from the previous step were also combined.

The separation was tried again (500mg sample) using a C-8 (Lobar) column with 60% CH<sub>3</sub>OH/H<sub>2</sub>O. Thirty five fractions were collected. The first was 95ml, the next thirty-three were each 20ml and the final fraction was a methanol strip (200ml). The column effluent for every second fraction was assayed, and the active fractions combined (fractions 15 to 35 exhibited antiviral activity).

The Lobar separation was repeated four more times (500mg aliquots of the active material). The active material from each of these separations was then combined as sample T10 (1.6g).

### ***Exploratory Chromatography of T10***

A small quantity (22 $\mu$ g) of the active material (T10) from the previous step was separated by HPLC (C-18 Econosphere 4.5mm x 250mm, 55% CH<sub>3</sub>OH/H<sub>2</sub>O at 1.5ml/min, UV 205nm). The chromatogram was a poorly resolved envelope indicating a complex mixture. Five fractions were collected (3.6ml, 2.4ml, 1.8ml, 2.1ml, 2.1ml) and assayed directly. Fractions two and four showed strong antiviral

activity and fractions one and three were mildly cytotoxic (F1 --,++/C3 40µl; F2 WW,+++,+++/C3 40µl; F3 --,++/C3 40µl; F4 WW,-,+ 40µl; F5 --,-, 40µl).

The small scale separation of T10 (1mg) was repeated using a new C-18 column (C-18 Econosphere 4.5mm x 250mm, 62.5% CH<sub>3</sub>OH/H<sub>2</sub>O at 1.5ml/min, UV 210nm), and 1mg of sample. Twelve fractions were collected (5.7ml, 0.9ml, 0.6ml, 0.75ml, 0.6ml, 0.5ml, 1.5ml, 0.9ml, 1.5ml, 1.2ml, 5.4ml, 4.8ml), with volumes being selected so as to isolate major peaks as far as possible. The antiviral activity was concentrated in fractions eight, nine and ten, which exhibited a characteristic fishy odour, while most of the other fractions exhibited some cytotoxic activity (F1 ?,nd,WW/C3/C1 40µl; F2 --,nd,+/C3 40µl; F3 --,nd,- 40µl; F4 ?,nd,+++/C3 40µl; F5 --,nd,- 40µl; F6 ++,nd,++/C3 40µl; F7 ++,nd,++/C3 5µl; F8 ++,nd,+/C3 40µl; F9 ++,nd,++/C3 5µl; F10 WW,nd,+/C3 40µl; F11 ?,nd,+++ 5µl; F12 --,nd,- 40µl).

### ***Trial Separations Using Cation-exchange***

An aliquot of sample T10 was spotted onto carboxymethyl cellulose TLC paper and eluted with 1+1 methanol/pH 4 buffer (aqueous 0.05M acetic acid/aq. ammonia). Two spots were visible. A spot at R<sub>f</sub> 0.85 gave a pale yellow spot when visualised by iodine vapour, a weak orange spot with ninhydrin, and a dark green spot with phosphomolybdic acid. A second spot at R<sub>f</sub> 0.15 gave a positive ninhydrin reaction, but was not visible with iodine or phosphomolybdic acid. To check that the retained ninhydrin material could be removed from the TLC paper another chromatogram was run, then dried *in vacuo*. The TLC paper was redeveloped using a 1+1 methanol/pH 10 buffer (aqueous 0.05M (C<sub>2</sub>H<sub>5</sub>)<sub>3</sub>N/TFA), and it was found that the ninhydrin positive was close to the solvent front.

The ion-exchange separation was repeated with about 0.5mg of sample T10. The active mixture was applied in a band 40mm long and eluted over a distance of 55mm. The solvents and buffer were removed *in vacuo* and the TLC paper was cut into seven strips (at R<sub>f</sub> 0.06, 0.29, 0.43, 0.61, 0.76, 0.85). A 5mm long piece of each strip was dropped directly into the antiviral assay well, however only cytotoxic activity was observed.

The remainder of the strips were divided into two groups about  $R_f$  0.43, and the absorbed material was washed off with methanol/pH 10 buffer (5ml). The solvents were removed, and the two samples made up to 1ml for assay. Only the high  $R_f$  material was found to be active (+++,nd,++/C3 5 $\mu$ l).

### Chemical Modification of Sample T10

A substantial excess of an ether solution of diazomethane<sup>156</sup> was added to each of two aliquots of sample T10 (2mg each). One sample was allowed to react for one minute before being quenched with acetic acid (50%), while the other was allowed to react for sixty minutes. The solvents were removed *in vacuo*. In each case the antiviral activity was still present (WW,WW,+ /C1 2 $\mu$ g; WW,WW,+ /C3 2 $\mu$ g). Analysis of the two reaction mixtures by silica gel TLC (10% CH<sub>3</sub>OH/CHCl<sub>3</sub>, visualised by phosphomolybdic acid) gave identical chromatograms with major spots at  $R_f$  0.3, 0.4, 0.63 and 0.8. Analysis of T10 under identical conditions showed major spots at  $R_f$  0.22, 0.28 and 0.32, and there was a streak of ninhydrin positive material from the baseline to  $R_f$  0.18

Hydrolysis of the methylation reaction products (200  $\mu$ l of 70% CH<sub>3</sub>OH/H<sub>2</sub>O and 1% KOH for 3 hr) yielded material with no antiviral activity. Analysis by TLC under the above conditions showed one major spot ( $R_f$  0.19).

A third aliquot of sample T10 (2mg) was acetylated with acetic anhydride (0.5ml) and pyridine (0.5ml) under nitrogen for 18 hours. The reagents were removed under reduced pressure. Analysis by silica gel TLC, using the above conditions, revealed six major spots ( $R_f$  0.79, 0.66, 0.55, 0.45, 0.05, 0.00) and there was a ninhydrin positive spot at  $R_f$  0.68. No antiviral activity was detected in the products.

---

<sup>156</sup> Prepared from N-methyl-N-nitrosotoluene-*p*-sulphonamide (2.1g) in ether (30ml) and KOH (0.4g) in ethanol (10ml), as described in Fieser, F.F. and Fieser, M., *Reagents for Organic Synthesis*, 191 (John Wiley and Sons : New York 1967).

The reactions products were hydrolysed under the above conditions. No antiviral activity was present in the products. Six spots were observed on the silica gel TLC ( $R_f$  0.81, 0.62, 0.54, 0.37, 0.23).

### ***Separation of Sample T10 by Semi-prep Scale HPLC***

A portion of sample T10 (about 76mg) was injected onto a reverse-phase semi-prep column (C-18 Econosphere 10mm x 250mm, 72.5% CH<sub>3</sub>OH/H<sub>2</sub>O at 5ml/min, UV 210nm). The sample was chromatographed 7 mg at a time and four fractions were collected (20ml, 25mg; 11ml, 6mg; 24ml, 11mg; 83ml, 14mg). The antiviral assay indicated the active material was contained in fractions two and three, while fraction four exhibited some cytotoxic activity (F2 WW,WW,+/C7 0.1µg; F3 WW,WW,- 0.1µg; F4 --,+++ 0.25µg).

Once suitable separation conditions had been established another 850mg of T10 was chromatographed. The antiviral activity was contained within fraction three, referred to as T11 (WW,WW,- 0.5µg).

### ***Small Scale Gel Permeation Chromatography of T11***

Fractogel PGM2000 (120g, Merck Art 9355) was swelled (18hr) in methanol before being slurry packed into a glass column (17mm x 340mm, LKB). Polyethylene glycol (M 4000) and benzophenone were run, with methanol as the eluting solvent (1ml/min) and UV detection (206nm), to obtain approximate values for  $V_0$  (50ml) and  $V_i$  (110ml).

Using the gel permeation column described in the previous paragraph, sample T11 (60mg) was chromatographed (CH<sub>3</sub>OH at 0.6ml/min; UV 206nm), and after the void volume had eluted, thirty-six fractions (6ml) were collected. Fractions eleven to seventeen were strongly antiviral, while the remaining fractions were mostly inactive or weakly cytotoxic (F11-15 WW,WW,- 10µl; F16 ++,WW,±/C6 10µl; F17 +++,WW,- 10µl; F18 +,WW,±/C6 10µl; F19 +.+++, - 10µl). Fractions ten to seventeen were combined (24mg) as sample T12.

The remainder of sample T11 (2 x 60mg) was chromatographed using the same gel permeation system. After the void volume (50ml) had been discarded thirty-one fractions were collected. The antiviral fractions, nine to sixteen, were combined (as sample T13). Fractions seventeen to thirty-one were cytotoxic, with some antiviral activity. (F9 WW,++,- 20µl; F10-12 WW,WW,- 20µl; F13 WW,WW,++/C3 20µl; F14 ?,WW,++/C3/C7 20µl; F15 +++,+++/C3 20µl; F16 ++,?,+/C6 20µl; F17-23 ?,?,++/C6/C7/C3 20µl; F27 ?,?,+/C7 20µl; fractions 16 onward had diffuse cytotoxicity and exhibited viral cytopathic effects).

### **HPLC of Sample T13**

Sample T13 was evaluated with two HPLC normal phase solvent systems, on a cyanopropyl column (CN Econosphere 4.5mm x 250mm) with UV detection (245nm). Methanol/chloroform mixtures (20%, 10%, 5% and 1% CH<sub>3</sub>OH) gave no retention, while some retention was observed with pure methanol. Pet. ether/chloroform mixtures (20%, 5%, 2% and 1% pet. ether) gave poor resolution and peak shapes. Assay of effluent collected from the experiment run in pure methanol indicated that the antiviral material (WW,WW,± 2µg) could be eluted under normal phase conditions.

In addition, two reverse-phase solvent systems were tried with a C-18 column (C-18 Econosphere 4.5mm x 250mm) and UV detection (210nm). The first system was methanol/water, for which the best separation was obtained with 70% methanol, although the chromatogram was a poorly resolved envelope. Five fractions (4.5ml, 2.1ml, 2.1ml, 3.3ml, 10.5ml) were collected, from an injection of sample T13 (50µg). The second third and fourth fractions had strong antiviral activity (F2,3,4 WW,WW,+/C7 1µg).

An almost identical chromatogram, and antiviral (F2,3 WW,WW,+/C7 1µg) result was obtained using an acetonitrile/water (50%) solvent system.

**Silica Gel Chromatography of T12 and T13**

An aliquot of sample T13 (10mg) was applied as a dichloromethane solution to a silica gel column (2.5g Davisil, 10mm x 100mm) and eluted with dichloromethane/methanol mixtures. Twelve fractions (4ml) and a strip (30ml) were collected (CH<sub>2</sub>Cl<sub>2</sub>, 0mg; CH<sub>2</sub>Cl<sub>2</sub>, 0mg; CH<sub>2</sub>Cl<sub>2</sub>, 0.2mg; 2% CH<sub>3</sub>OH/CH<sub>2</sub>Cl<sub>2</sub>, 0.1mg; 2% CH<sub>3</sub>OH/CH<sub>2</sub>Cl<sub>2</sub>, 0mg; 2% CH<sub>3</sub>OH/CH<sub>2</sub>Cl<sub>2</sub>, 0.3mg; 5% CH<sub>3</sub>OH/CH<sub>2</sub>Cl<sub>2</sub>, 0.5mg; 5% CH<sub>3</sub>OH/CH<sub>2</sub>Cl<sub>2</sub>, 0.5mg; 5% CH<sub>3</sub>OH/CH<sub>2</sub>Cl<sub>2</sub>, 0.3mg; 10% CH<sub>3</sub>OH/CH<sub>2</sub>Cl<sub>2</sub>, 0.4mg; 10% CH<sub>3</sub>OH/CH<sub>2</sub>Cl<sub>2</sub>, 0.7mg; 10% CH<sub>3</sub>OH/CH<sub>2</sub>Cl<sub>2</sub>, 9.5mg; stripped with CH<sub>3</sub>OH, no reliable weight). Antiviral activity was found in fractions eight to twelve, but concentrated in fractions eight and nine (F8 WW,WW,+/C7 20ng; F9 +,+,+,+/C7 20ng; F10 ++,WW,+/C7 200ng; F11 WW,WW,+/C7 2μg; F12 +,+,+,+/C7 2μg).

The remainder of T12 and T13 were combined (35mg) and chromatographed on silica gel (4.5g Davisil 30-70μ, 10mm x 150mm). Fourteen fractions (8ml, except for 13 and 14) were collected (CH<sub>2</sub>Cl<sub>2</sub>, 0mg; CH<sub>2</sub>Cl<sub>2</sub>, 0mg; CH<sub>2</sub>Cl<sub>2</sub>, 0.2mg; 2% CH<sub>3</sub>OH/CH<sub>2</sub>Cl<sub>2</sub>, 0mg; 2% CH<sub>3</sub>OH/CH<sub>2</sub>Cl<sub>2</sub>, 0mg; 2% CH<sub>3</sub>OH/CH<sub>2</sub>Cl<sub>2</sub>, 0.3mg; 5% CH<sub>3</sub>OH/CH<sub>2</sub>Cl<sub>2</sub>, 1.7mg; 5% CH<sub>3</sub>OH/CH<sub>2</sub>Cl<sub>2</sub>, 1.0mg; 5% CH<sub>3</sub>OH/CH<sub>2</sub>Cl<sub>2</sub>, 1.5mg; 10% CH<sub>3</sub>OH/CH<sub>2</sub>Cl<sub>2</sub>, 0.4mg; 10% CH<sub>3</sub>OH/CH<sub>2</sub>Cl<sub>2</sub>, 0.5mg; 10% CH<sub>3</sub>OH/CH<sub>2</sub>Cl<sub>2</sub>, 0.4mg; 50 ml 50% CH<sub>3</sub>OH/CH<sub>2</sub>Cl<sub>2</sub>, 23mg; 50ml 90% CH<sub>3</sub>OH/CH<sub>2</sub>Cl<sub>2</sub>, 0mg). Antiviral activity was detected in fractions eight to thirteen (F1 to F6 --- 2μg; F8 WW,WW,+/C7 20ng; F9 to F12 WW,WW,+/C7 2μg; F13 ++,nd,+/C7 2μg; F14 --- 2μg).

The combined active fractions from this, and the previous column (fractions 7, 8, 9, 10, 11, 12 from the first column, and 7, 8, 9, 10, 11 from the second, 6mg) were re-chromatographed on silica gel (4.5g Davisil 30-70μ, 10mm x 150mm). Thirteen fractions (8ml) were taken (F1 to F4 CH<sub>2</sub>Cl<sub>2</sub>; F5 to F8 2% CH<sub>3</sub>OH/CH<sub>2</sub>Cl<sub>2</sub>; F9 to F12 5% CH<sub>3</sub>OH/CH<sub>2</sub>Cl<sub>2</sub>; stripped with 50ml 50% CH<sub>3</sub>OH/CH<sub>2</sub>Cl<sub>2</sub>). For antiviral assay it was assumed that fractions six to thirteen each contained 0.5mg. The activity was concentrated in fraction seven (F7 WW,WW,+/C7 20ng; F8 to F12 WW,WW,+/C7 2μg; F13 ++,-,+/C7 2μg).

Fractions seven to eleven were combined as sample T14 (4.0mg).

#### **HPLC of Sample T14**

Sample T14 was chromatographed by reverse-phase chromatography (C-18 Econosphere, 4.5mm x 250mm; 65% MeOH/H<sub>2</sub>O at 1.5ml/min; UV 215nm). Repeated injections of 100µg were made and thirty-five fractions (nominally F1, F2 1.5ml, F3 to F34 0.5ml, F35 15ml) collected. The fractions were made up to the same volume (10ml) for assay. Antiviral activity was present in three groups of fractions: fractions twenty to twenty-five (F20 WW,+++,+/C7 10µl; F21 to F24 WW,WW,+/C7 10µl; F25 ++,+++,+/C7); fractions twenty-eight and twenty-nine (F28, F29 +++,WW,+/C7 10µl); fractions thirty-two to thirty-nine (F32 to F39 WW,WW,+/C7 10µl). Selected fractions were weighed by evaporating their chloroform solutions, under a stream of nitrogen, onto tared microscope cover slips, working in a clean room (F19 40µg; F20 50µg; F21 <10µg; F22 110µg; F23 40µg; F24 <10µg; F33 60µg; F34 60µg; F35 670µg). The weighing method was tested using a cholesterol solution of known concentration (5mg/ml) and found to be reproducible to less than 10µg at a mass of 50µg.

#### **HPLC of T14 Fractions**

The active components of fractions twenty-two, twenty-three and thirty-three to thirty-five, referred to as T14.22, T14.23 and T14.33 to T14.35 respectively, were further purified by reverse-phase HPLC.

A few micrograms of each of these fractions were first chromatographed to establish the conditions for the bulk separations: C-18 Adsorbosphere, 4.5mm x 250mm; 50% CH<sub>3</sub>CN/H<sub>2</sub>O, 1.5ml/min; UV 190nm. Total amount of each of these samples was chromatographed (except for T14.35 200µg), using four injections for each. The fractions were collected as described below, and assayed directly.

T14.22 - F1 7.8ml (WW,WW,+/C7 40µl); F2 1.5ml (-,-,- 40µl); F3 1.2ml (-,-,- 40µl); F4 0.9ml (-,-,- 40µl); F5 4.5ml (-,-,- 40µl).



T14.23 - discarded 0.27ml; F1 1.5ml (-,-,- 40 $\mu$ l); F2 3.6ml (-,-,- 40 $\mu$ l); F3 0.9ml (WW,WW,+ /C7); F4 2.4ml (-,-,- 40 $\mu$ l); F5 2.1ml (-,-,- 40 $\mu$ l); F6 10ml (-,-,- 40 $\mu$ l);

T14.33 - F1 8.1ml (WW,WW,+ /C7 40 $\mu$ l); F2 2.4ml (-,-,- 40 $\mu$ l); F3 1.5ml (-,-,- 40 $\mu$ l); F4 6ml (-,-,- 40 $\mu$ l).

T14.34 - F1 8.7ml (WW,WW,+ /C7 40 $\mu$ l, -,-,- 5 $\mu$ l); F2 1.8ml (WW,WW,+ /C7 5 $\mu$ l); F3 1.5ml (WW,WW,+ /C7 5 $\mu$ l); F4 6ml (WW,WW,+ /C7 40 $\mu$ l, -,-,- 5 $\mu$ l).

T14.35 - F1 7.5ml (WW,WW,+ /C7 5 $\mu$ l); F2 3.3ml (WW,WW,+ /C7 5 $\mu$ l); F3 1.8ml (WW,WW,+ /C7 40 $\mu$ l, -,-,- 5 $\mu$ l); F4 1.5ml (+,+,+ /C7 40 $\mu$ l); F5 2.1ml (+,+,+ /C7 40 $\mu$ l); F6 3.9ml (WW,+,+,+ /C7 40 $\mu$ l); F7 34.5ml (WW,WW,+ /C7 40 $\mu$ l, +,+,+ /C7 5 $\mu$ l).

### Mass Spectrometry of Selected Fractions

Attempts were made to obtain low resolution DCI spectra, using Finnegan 4500 spectrometer, with ammonia and methane as reagent gases. The probe was heated at 20°/s. The following positive-ion data was recorded, scanning from  $m/z$  500 to 700 with ammonia as the reagent gas. T14.19  $m/z$  554, 552, 526, 524; T14.20  $m/z$  628, 612, 550; T14.22 no ions observed; T14.23  $m/z$  628, 610, 512; T14.24 no ions observed; T14.33  $m/z$  662; T14.34  $m/z$  612; T14.35  $m/z$  586. Ions at  $m/z$  636 and 619 were observed for T14.23 and T14.34 respectively, in the positive ion DCI/ND<sub>3</sub> spectra. Negative ion DCI spectra were recorded with methane as the reagent gas as follows: T14.23  $m/z$  610; T14.33  $m/z$  644; T14.34  $m/z$  594.

Positive-ion high resolution FAB spectra were obtained for the following samples, on a VG7070E mass spectrometer with Xenon and a thioglycerol matrix.<sup>157</sup> T14..23  $m/z$  608.3862 no 610 ion was observed; T14.33  $m/z$  667.2909 (C<sub>32</sub>H<sub>49</sub>O<sub>11</sub>ClNa requires 667.2814); T14.34  $m/z$  617.3311, 612.3716, 577.3328 (C<sub>32</sub>H<sub>50</sub>O<sub>10</sub> requires 594.3404; C<sub>32</sub>H<sub>50</sub>O<sub>10</sub>Na requires 617.3302; C<sub>32</sub>H<sub>49</sub>O<sub>9</sub> requires 577.3376).

---

<sup>157</sup> Pannell, L.K., personal communication. Spectra were by Dept of Chemistry, University of Minnesota

### Tests for Tedanolide

The  $^{13}\text{C}$ -NMR spectra of sample T12, and of the other sets of combined gel permeation fractions from the same run, were compared to that of tedanolide (13).<sup>158</sup> The spectra of these fractions indicated they were mixtures, and none of the singlet resonances characteristic of tedanolide were observed.

Tedanolide gives a characteristic pale yellow colour reaction when a silica gel TLC plate ( $R_f$  0.38 with 8% MeOH/ $\text{H}_2\text{O}$ ) is visualised with vanillin/sulphuric (5% vanillin/conc. sulphuric acid).<sup>159</sup> Analysis of the three set of combined gel permeation fractions revealed no spots consistent with the presence of tedanolide.

### Tests for Bryostatin

The  $^1\text{H}$ -NMR spectrum of was for the characteristic singlet resonances (for example H20 and H34 at approximately  $\delta_{\text{H}}$  5.9 and 5.16 ppm) reported for bryostatins, but none were observed.

Bryostatin was also reported to give characteristic TLC purple colour reaction with an anisaldehyde visualising agent (1ml  $\text{H}_2\text{SO}_4$ , 0.1ml anisaldehyde, 100ml glacial acetic acid)<sup>160</sup> at  $R_f$  0.7 on silica gel (10% MeOH/ $\text{H}_2\text{O}$ ). Under these conditions five red-brown spots were observed ( $R_f$  0.40, 0.33, 0.27, 0.17, 0.0).<sup>161</sup>

### Sterols

---

Nine samples of *Tedania* were collected from Kaikoura. The samples were taken from individual sponges, and bagged before being brought to the surface. Sub-samples of approximately 2g were extracted with methanol/toluene (3+1), and submitted for antiviral assay, with the following results: 5K17-4 WW,WW,++;

---

158 Supplied by Schmitz, F. Personal communication

159 Gunasekera, S., personal communication.

160 CRC Handbook Series in Chromatography, Section A: Data and Principles, Vol 2, Ed Zweig, G., Sherm, J. (CRC Press : Florida 1972).

161 Pettit, G.R., Kamano, Y., Herald, C.L. and Tozawa, T., *Can. J. Chem.*, **63**, 1204 (1985).

5K17-5 -,-,-; 5K17-9 +++,WW,++; 5K17-10 WW,WW,++; 5K17-11 +++,+++,++; 5K17-12 WW,+++,++; 5K17-13 WW,WW,++; 5K17-14 +++,+++,++; 5K17-15 WW,+++,++. These samples were all identified as *T. connectens*, except for 5K17-4 which was *T. diversirhaphidiophora*.

The samples (10mg) were derivatised with 200µl neat trimethylsilylimidazole (Pierce) at 100°C for one hour. The derivatives were analysed directly by capillary GC: 50m DB-1 (J&W) column; column temperature 230°C; injector 290°C; detector FID at 310°C. The carrier gas was helium at 1ml/min. Attempts were made to record the mass spectra of the sterols with a Carlo Erba MFC-500 gas chromatograph connected to a Kratos MS-80 mass spectrometer.

Peaks were observed, relative to cholesterol, at 0.646, 0.901, 1.000, 1.081, 1.102, 1.257, 1.283, 1.604 and 1.666.

### Collection Record

---

The following is a record of the *Tedania* species collected by the marine chemistry group at the University of Canterbury, together with the pre-screening assay results.

*Tedania connectens*: 821208/2/003 (++++,+-); 821214/1/004 (+,-,-); 830731-5 (++++,?,+++); 830801-1 (-,-,+); 830801-3 (-,-,-); 831004-2 (-,-,+); 831004-3 (++++,-); 831004-5 (+,-,-); 831129-30 (++++,-); 831129-31 (+,-,-); 831130-20 (-,-,-); 831130-47 (++++,-); 831202-11 (++++,+); 831202-12 (-,-,-); 840209-10 (++++,-); 840209-9 (++++,-); 840209-11 (++++,-); T459-3 (++++,-); 5K3-11 (?,?,+++); 5K17-5 (-,-,-); 5K17-9 (++++,WW,++); 5K17-10 (WW,WW,++); 5K17-11 (++++,++); 5K17-12 (WW,++,-); 5K17-13 (WW,WW,++); 5K17-14 (++++,++); 5K17-15 (WW,++++); 5P2-24 (++++,++); 5P3-1 (-,-,-); 831129-29 (++++,++); 5P2-16 (-,-,+); 6P6-1 (WW,?,WW); 6WI1-8 (WW,-,+).

*Tedania diversirhaphidiophora*: 5K17-4 (WW,WW,++); 5NP2-9 (?,?,WW); 6AKI5-4 (++++,++); 6AKI6-4 (WW,?,++); 4L4-20 (-,-,-);

*Tedania battershilli*: 821210/1/012 (-,-,-); 4L3-36 (-,-,-).

*Tedania* n. sp. A: 831202-10 (-,+,-).

*Tedania* n. sp. B: PML1-6 (+++,+++,+++); 5P2-16 (-,-,++).

---

## Thyrsiferol

---

### Isolation of Thyrsiferol

A frozen sample of *Laurencia thyrsifera* (2.1kg, voucher 85K22-1) was homogenised in methanol (1.5l) and filtered. The residue was re-extracted with methanol (3 x 1.5l) then with dichloromethane (3 x 1.5l) and the combined extracts were then reduced, *in vacuo*, until the organic solvents had been removed (total volume 300ml, estimated solids of approximately 100g).

The extract was slurried with reverse-phase material (C-18, 100g) and applied to a column (400g C-18, 90mm x 150mm). Six fractions were eluted with a steep, stepped gradient (H<sub>2</sub>O, 2l, weight not measured; 50% CH<sub>3</sub>OH/H<sub>2</sub>O, 2l, 5.0g; 80% CH<sub>3</sub>OH/H<sub>2</sub>O, 1l, 1.31g; 80% CH<sub>3</sub>OH/H<sub>2</sub>O, 1l, 1.79g; CH<sub>3</sub>OH, 2l, 7.7g; strip (CH<sub>3</sub>OH/CH<sub>2</sub>Cl<sub>2</sub>/pet. ether), 2l 2.14g), and analysed by TLC (silica gel, developed with 10% CH<sub>3</sub>OH/H<sub>2</sub>O, visualised with anisaldehyde dip).<sup>162</sup> Thyrsiferol, which gives a distinctive yellow spot at R<sub>f</sub> 0.5 under these conditions, was found in fraction five.

Fraction five was rechromatographed on a silica gel (200g, Grace 923) column (40mm x 500mm). The sample (7.7g) was coated onto a small quantity of silica and added to the column as a powder, before being eluted with pet. ether and ethyl acetate. Six fractions were collected (33% EtOAc/pet. ether, 800ml, 3.5g; 50% EtOAc/pet. ether, 400ml, 550mg; 50% EtOAc/pet. ether, 400ml, 300mg; 66% EtOAc/pet. ether, 400ml, 210mg; 66% EtOAc/pet. ether, 400ml, 570mg; 20% CH<sub>3</sub>OH/EtOAc, 800ml, 830mg). The thyrsiferol was located in fraction five by <sup>1</sup>H-NMR.

A steel MPLC column (22mm x 350mm) was packed with silica gel (Davisil, 20-30μ) using a chloroform slurry. A portion of fraction five (400mg) was dissolved

---

162 CRC Handbook Series in Chromatography, Section A: Data and Principles, Vol 2, Ed. Zweig, G., Sherm, J. (CRC Press : Florida 1972).

in 57% ethyl acetate/pet. ether, then injected onto the column. Twenty-four fractions (20ml) were eluted with the same solvent mixture (5.5ml/min), and analysed by silica gel TLC (developed with 10% CH<sub>3</sub>OH/CHCl<sub>3</sub>, visualised with anisaldehyde dip). The fractions nine to fifteen were found to contain thyrsiferol (200mg). The remainder of fraction five from the previous step was chromatographed in the same manner, except the eluting solvent was 43% ethyl acetate/pet. ether. The thyrsiferol-containing fractions were combined.

Pure (by <sup>1</sup>H-NMR) thyrsiferol was obtained by recrystallisation from 50% ethyl acetate/pet. ether. Thyrsiferol exhibited antiviral activity (+++, ++, + 1.0µg, ++, -, - 0.1µg), P388 activity (36 ng/ml) and mild activity against *Candida albicans* (5mm zone at 60µg/disk).

#### ***Oxidation of Thyrsiferol - Jones' Reagent***

Jones' reagent was prepared by dissolving chromium trioxide (26g) in concentrated sulphuric acid (23ml), then adding the solution slowly to water (100ml). The fresh oxidising reagent was added to an acetone (500µl) solution of thyrsiferol (5mg), until the red colour persisted. Sodium metabisulphite (CP) solution (saturated) was added until the colour was discharged. The reaction mixture was added to water (10ml), then extracted with dichloromethane (3 × 10ml). The dichloromethane solution was washed with water (1 × 10ml) and dried (anhydrous Na<sub>2</sub>SO<sub>4</sub>).<sup>163</sup> Analysis of the reaction mixture by TLC (silica gel, developed with 66% EtOAc/pet. ether), visualised with anisaldehyde) revealed the presence of at least seven compounds (R<sub>f</sub> 0.14, 0.22, 0.29, 0.46, 0.56, 0.65, 0.74). The spots were yellow or brown, except for the spot at R<sub>f</sub> 0.29, which was bright pink. Under the same conditions thyrsiferol eluted at R<sub>f</sub> 0.14. The reaction mixture was submitted for antiviral assay (-, +, - 0.1µg)

---

163 Fieser, L.F. and Fieser, M., *Reagents for Organic Synthesis*, 142, (John Wiley & Sons : New York 1967).

### **Acid Treatment of Thyransferol**

Perchloric acid (2 drops, Univar AR) was added to thyransferol (10mg) in acetone (1ml, M&B Proanalys). After five minutes the reaction mixture was neutralised with aqueous sodium bicarbonate, extracted with chloroform (3 x 20ml) and dried ( $\text{MgSO}_4$ ). Analysis by TLC, as described above, indicated that no change had taken place. The reaction was repeated, allowing one and a half hours before neutralisation. Six spots were revealed by TLC analysis ( $R_f$  0.14, 0.22, 0.46, 0.56, 0.65, 0.74). The reaction products exhibited no antiviral activity at 1.0 $\mu\text{g}$ /disk

### **Oxidation of Thyransferol - Pyridinium Chlorochromate**

A solution of thyransferol (20mg) in anhydrous dichloromethane was added to a dichloromethane (2ml, anhydrous) solution of pyridinium chlorochromate (12.9mg) and sodium acetate (15mg, anhydrous). After stirring for ninety minutes, the reaction mixture was added to dry ether (20ml), and the solid residue was washed with more dry ether (3 x 5ml). The combined ether solution was filtered through Florisil (BDH).<sup>164</sup> Analysis by TLC (silica gel, developed with 66% EtOAc/pet. ether, visualised with a spray of 5% vanillin/conc.  $\text{H}_2\text{SO}_4$ , brown spots were observed), revealed the presence of at least three compounds ( $R_f$  0.12, 0.51, 0.61) other than the major component, thyransferol ( $R_f$  0.22). The solution IR ( $\text{CCl}_4$ ) showed a strong absorbance at  $\nu$  1720 $\text{cm}^{-1}$ . The reaction products displayed some antiviral activity (+,?, + 1.0 $\mu\text{g}$ )

### **Oxidation of Thyransferol - Swern Reaction**

Oxalyl chloride (1 $\mu\text{l}$ , Aldrich, freshly opened) and dichloromethane (30 $\mu\text{l}$ , anhydrous) were placed in a Reacti-vial, which was then sealed and cooled in a dry ice/propan-2-ol bath. Dimethylsulphoxide (1.7 $\mu\text{l}$ , Riedel-De Haën), in dichloromethane (5 $\mu\text{l}$ ), was added, by syringe, through the septum. After five minutes thyransferol (5mg), dissolved in dichloromethane (10 $\mu\text{l}$ ), was added. The

---

164 Piancatelli, G., Scettri, A., D'Auria, M., *Synthesis*, 245 (1982).

reaction was allowed to proceed for thirty minutes, before triethylamine (7 $\mu$ l) was added. After a further five minutes the mixture was allowed to warm before opening. Analysis of the reaction mixture, by TLC, indicated that no reaction had taken place.<sup>165</sup>

#### **Oxidation of Thyrsiferol - Moffat Reaction**

Thyrsiferol (4.5mg) was dissolved in benzene (75 $\mu$ l), containing dimethyl sulphoxide (15 $\mu$ l, anhydrous), pyridine (1 $\mu$ l, freshly distilled) and dicyclohexylcarbodiimide (6mg). The Reacti-vial, containing the reagents, was sealed and flushed with nitrogen, before trifluoroacetic acid (1 $\mu$ l) in benzene (5 $\mu$ l) was added. After standing at room temperature for twenty-four hours, the reaction mixture was added to ethyl acetate (10ml) and washed with water (3 x 10ml). Examination of the reaction products by TLC (silica gel, developed with 10% CH<sub>3</sub>OH/CHCl<sub>3</sub>) indicated that no reaction had occurred.<sup>166</sup>

#### **Preparation of Thyrsiferyl 18-Mesylate**

Mesyl chloride (5 $\mu$ l) was added to a pyridine (250 $\mu$ l) solution of thyrsiferol (10 $\mu$ g). Crystals of pyridine hydrochloride were formed immediately. The mixture was stirred at room temperature for thirty minutes, before dichloromethane (10ml) was added. The reaction mixture was then washed with water (2 x 10ml) in a Mixxor extractor. The organic layer was dried (anhydrous MgSO<sub>4</sub>) and the solvents removed, *in vacuo*. The last traces of pyridine were removed by repeatedly adding benzene (2ml) and then rotary evaporation of the solution to dryness.

Only one spot ( $R_f$  0.40), apart from thyrsiferol ( $R_f$  0.27) was observed in the TLC (silica gel, developed with 5% CH<sub>3</sub>OH/CHCl<sub>3</sub>, visualised with vanillin/H<sub>2</sub>SO<sub>4</sub>). The <sup>1</sup>H-NMR spectrum was consistent with the formation of a 18-mesylate, with the H18 proton resonance being observed at  $\delta_H$  4.6ppm, and a sharp singlet at  $\delta_H$

---

165 Mancuso, A.J., Shui-Lung Huang and Swern, D., *J. Org. Chem.*, **43**, 2480 (1978).

166 Pfitzner, K.E. and Moffat, J.G., *J. Am. Chem. Soc.*, **87**, 5670 (1965).



3.05ppm. The spectrum also showed almost complete conversion of thyrsiferol to the mesylate.

#### ***Hydrolysis of Thyrsiferyl 18-Mesylate***

Calcium hydroxide (3mg, CP) and water (10 $\mu$ l) was added to an acetone solution (200 $\mu$ l) of thyrsiferyl 18-mesylate (5mg). Examination of the reaction mixture by TLC indicated that no reaction had occurred after twenty-four hours. Further water (15 $\mu$ l) and calcium hydroxide (3mg) were then added to the reaction mixture. No change was observed by TLC after one hour, but after thirty hours more than seven compounds, other than the mesylate ( $R_f$  0.45) were observed ( $R_f$  0.10, 0.27, 0.37, 0.57, 0.67, 0.78, 0.88).

The hydrolysis was repeated with further aliquots of the mesylate (1.5mg). Five samples were dissolved in acetone, as above, and 15 $\mu$ l, 30 $\mu$ l, 60 $\mu$ l, 120 $\mu$ l of water was added, respectively. After two days, at room temperature, no reaction was observed. The reaction mixtures were then warmed to 45°C for twelve hours. The latter two reactions mixtures were found to be mixtures similar to that obtained in the first experiment.

#### ***Mitsunobu Reaction***

A trial reaction with cholesterol as a substrate, was performed. Cholesterol (5mg, BDH), triphenyl phosphine (3mg, BDH) and acetic acid (1 $\mu$ l, CP) were dissolved in tetrahydrofuran (150 $\mu$ l, Univar AR). Diethyl azodicarboxylate (2 $\mu$ l) was added, and the reaction mixture was stirred for twenty-four hours. Direct TLC analysis (silica gel, developed with CH<sub>3</sub>OH/CHCl<sub>3</sub>, visualised with anisaldehyde dip) of the reaction mixture indicated a clean, but incomplete reaction (cholesterol at  $R_f$  0.42, product at  $R_f$  0.76).<sup>167</sup>

---

167 Bose, A.K., Bansil Lal, Hoffman, W.A. III, Manhas, M.S., *Tetrahedron Lett.*, **18**, 1619 (1973).

Thyrsiferol (5mg) was dissolved in tetrahydrofuran (200 $\mu$ l), with triphenyl phosphine (4.2mg) and acetic acid (1mg). Diethyl azodicarboxylate (3.5mg,) in 50 $\mu$ l of tetrahydrofuran, was then added, and the mixture stirred for five days. The solvent was removed, under reduced pressure, and the reagents were removed by filtering a 43% ethyl acetate/ pet. ether solution of the products through a short Florisil column (10mm x 30mm). Analysis of the reaction mixture by TLC and  $^1\text{H}$ -NMR showed no evidence of any change.

### Acetylation of Thyrsiferol

Acetic anhydride (3 $\mu$ l) was added to a pyridine (200 $\mu$ l) solution of thyrsiferol (8mg). The acetic anhydride and pyridine were removed *in vacuo*, and the final traces of pyridine were removed by repeatedly adding benzene and then by rotary evaporation of the sample to dryness. A 10% methanol/ chloroform solution of the reaction products was passed through a silica gel column (0.7mm x 60mm) to yield pure (by  $^1\text{H}$ -NMR) thyrsiferyl 18-acetate (yield 90%).

### NMR Conditions

NMR spectra of thyrsiferol (8mg) and thyrsiferol 18-acetate (20mg) were obtained at 23°C on a Varian XL300 spectrometer (5mm broad-band switchable probe;  $^1\text{H}$  NMR at 300MHz and  $^{13}\text{C}$  NMR at 75MHz). The DEPT and COSY spectra were obtained using Varian standard pulse sequences. Both absolute value and double quantum filtered phase sensitive modes were used to obtain the COSY spectra (SW 1300Hz; 512 increments for F1 and F2; aquisition 0.395s; presequence delay 1s). The HETCOR spectra were obtained in absolute value mode using Varian pulse sequences ( $^{13}\text{C}$  SW 5600Hz,  $^1\text{H}$  SW 1300Hz; 128 increments of F1 and F2; aquisition 0.2s; presequence delay 1s). XCORFE<sup>168</sup> spectra were obtained with a T value of 0.128s followed by a T of 0.07s in a second experiment. Other parameters were the same as in the HETCOR experiment.

---

168 Reynolds, W.F., Hughes, D.W., Perpich-Dumont, M. and Enriquez, R.G., *J. Magn. Reson.*, **64**, 303 (1985).

### ***New Butenolide Dimers from Delisea elegans***

---

A substantial collection of *Delisea elegans* was made from St Kilda Rocks, in April 1983, and stored by freezing. The frozen alga (4kg) was air dried in darkness (300g dry weight), ground and extracted with ether, for 24 hours, in a Soxhlet apparatus. The resulting oil (10g) was chromatographed on a silica gel filtration column (50mm deep, 150mm dia. [sic], 350g Grace 923 silica gel), eluting with solvent mixtures ranging from pet. ether through to ether: pet. ether 1.5l; 0.5%, 5%, 10%, 20%, 50%, 100% ether, 1.0l each.

The three initial fractions (39mg) were shown to be heptadecane. Identification was by  $^{13}\text{C}$ -NMR.  $\delta_{\text{c}}$  32.04, 29.78, 22.70, 14.02; HREIMS  $m/z$  240.2819 ( $\text{C}_{17}\text{H}_{36}$ ) requires 240.2817. The identification was confirmed by capillary GC comparison with standard n-alkanes, using a semilog plot of retention time against chain length; 50m DB-1 (J&W) column at 200 $^{\circ}$ ;  $\text{C}_{16}$ , 3.69 min;  $\text{C}_{18}$  6.13 min;  $\text{C}_{19}$  8.26 min;  $\text{C}_{20}$  11.40 min;  $\text{C}_{22}$  22.77 min; unknown 4.67 min. A calculated retention time for  $\text{C}_{17}\text{H}_{36}$  was 4.69 min.

The fourth and fifth fractions (3.4g) were chromatographed by normal-phase HPLC (cyanopropyl; 0.1% propan-2-ol/hexane) to yield pure 3-butyl-4-bromo-5-(dibromomethylidene)-2(5H)-furanone (440mg) and a mixture of more polar compounds (85mg). Further HPLC of the mixture of polar compounds, under similar conditions, afforded two pure crystalline compounds (30mg, 15mg). Crystallisation was achieved by dissolving the material in pet. ether/ether and allowing the solvents to evaporate slowly at 4 $^{\circ}\text{C}$ . The structures of both compounds were determined by X-ray crystallography. X-ray data were collected using a Nicolet XRD P3 single crystal four circle diffractometer with Mo K $\alpha$  ( $\lambda$  0.71069 Å), at 173 K. The cell parameters were determined by a least-squares refinement of the setting angles of 25 accurately centred high angle reflections.

3,5',7',7',-Tetrabromo-1'-*A*-dibutyl-4'-(dibromomethylene)spiro[furan-2(5H),6'-[3]oxabicyclo[3.2.0]heptane]-2',5-dione: m.p. 105.5-106 $^{\circ}\text{C}$ ; IR ( $\text{CCl}_4$ ) 1830, 1800  $\text{cm}^{-1}$ ;  $^{13}\text{C}$ -NMR  $\delta_{\text{c}}$  165.46, 138.62, 137.59, 131.32, 81.33, 63.79, 61.70, 60.48, 37.55, 28.54,

26.28, 25.85, 22.60, 13.68. A number of resonances were not observed due to the small quantity of material available. UV  $\lambda_{\text{max}}$  acetonitrile ( $\epsilon$ ) 238nm (14900). Crystal data: –  $\text{C}_{18}\text{H}_{18}\text{Br}_6\text{O}_4$ , M 778, triclinic, space group P1, a 7.510(1), b 8.134(1), c 19.608(1) Å,  $\alpha$  99.12(1),  $\beta$  98.45(1),  $\gamma$  97.38(1)°, U 1155.59 Å<sup>3</sup>,  $D_c$  2.24 g cm<sup>-3</sup>, Z 2,  $\mu$  (Mo K $\alpha$ ) 103.47 cm<sup>-1</sup>. Approximate crystal dimensions 0.58 x 0.20 x 0.18mm; number of independent reflections measured 2679, number with  $I > 3\sigma(I)$  2091; an unique data set was collected using  $2\theta$ - $\omega$  scans to  $2\theta$  83°; F(000) 736; g 0.0011; R 0.046; ratio of transmission factors 1.984.

(Z,Z)-5,5'-(1,2-Dibromo-1,2-ethanediylidene)bis[4-bromo-3-butyl-2(5H)-furanone]: IR (CCl<sub>4</sub>) 1790 cm<sup>-1</sup>; <sup>13</sup>C-NMR (CDCl<sub>3</sub>)  $\delta_c$  164.85, 148.06, 138.29, 127.58, 102.16, 28.93, 25.56, 22.39, 13.66; UV  $\lambda_{\text{max}}$  acetonitrile ( $\epsilon$ ) 292nm (26000). Crystal data:  $\text{C}_{18}\text{H}_{18}\text{Br}_4\text{O}_4$ , M 618, triclinic, space group P1, a 7.728(1), b 10.030(1), c 14.048(2) Å,  $\alpha$  82.52(1),  $\beta$  81.45(1),  $\gamma$  86.75(1)°, U 1066.82 Å<sup>3</sup>,  $D_c$  1.93 g cm<sup>-3</sup>, Z 2,  $\mu$  (Mo K $\alpha$ ) 74.92 cm<sup>-1</sup>. Approximate crystal dimensions 0.16 x 0.52 x 0.09mm. Number of independent reflections measured 2795, number with  $I > 3\sigma(I)$  2276; an unique data set was collected using  $\omega$  scans to  $2\theta$  90°; F(000) 596; g 0.0013; R 0.059; ratio of transmission factors 1.827.

Calculations were performed on a Nova 4X computer using SHELXTL.<sup>168</sup>

---

168. Sheldrick, G.M., *SHELXTL User Manual*, Revision 4.1 (Nicolet XRD Corporation: Madison Wisconsin 1981).

### Crystal Structure of Eudistomin K

---

The data was collected with a Nicolet XRD P3 single crystal four circle diffractometer using Mo K $\alpha$  ( $\lambda$  0.71069 Å) radiation from a crystal monochromator. The cell parameters were determined from a least squares refinement of 22 accurately centred high angle reflections ( $19^\circ < \theta$ ). Data were corrected for Lorentz and polarisation effects. An empirical absorption correction, based on  $\psi$ -scan data, was applied. Crystal data. C<sub>21</sub>H<sub>19</sub>N<sub>3</sub>O<sub>2</sub>SBr<sub>2</sub>, M 535, crystal dimensions 0.8 x 0.4 x 0.1 mm, monoclinic, P2<sub>1</sub>, a 9.994(2), b 21.115(8), c 10.788(2) Å,  $\beta$  104.82(2), U 2200(2) Å<sup>3</sup>, D<sub>c</sub> 1.61 cm<sup>-3</sup>, Z 4, F(000) 1072,  $\mu$ (Mo K $\alpha$ ) 3.72 mm<sup>-1</sup>, absorption correction: max 0.755, min 0.414.  $\omega$ -scan intensity measurements ( $4^\circ < \theta$ ), at 133K, yielded 4374 unique reflections 3009 were judged observed ( $I > 3\sigma(I)$ ). Crystal stability was monitored by measuring three check reflections every 500 and no significant variations were observed.

The structure was solved using conventional heavy atom methods. Calculations were performed using IBM-PC (SHELXS), VAX (SHELX-76 and SHELXS) and NOVA 4X (SHELXTL) computers.<sup>169</sup> Hydrogen atoms were added at calculated positions, using a riding model with thermal parameters equal to 1.2U of their carrier atoms. All non-hydrogen atoms, except for the indolic carbon atoms, were refined anisotropically, with reflection weights of  $1/[\sigma^2(F)+0.00084(F^2)]$ , minimising the function  $\Sigma(F_o - F_c)$ . The absolute configuration was determined by *eta* refinement.<sup>170</sup> A value of  $\eta$  +0.95(3) was obtained from refinements with starting points of +1 and -1.

---

169. Sheldrick, G.M., *SHELXS. Program for Crystal Structure Solution* (University of Gottingen : Federal Republic of Germany), Sheldrick, G.M., *SHELXTL User Manual*, Revision 4.1 (Nicolet XRD Corporation : Madison, Wisconsin 1981), and Sheldrick, G.M., *SHELX-76 User Manual*.

170. Rogers, D., *Acta Cryst.*, A37, 734 (1981).

---

**MassCalc V3.06**

---

1.0 Introduction	152
1.1 The Atom/Fragment Data File	152
1.2 The Spectrum Data File	153
1.3 Printer and Plotters	154
2.0 MassCalc Commands	154
2.1 MassCalc Command Structure	154
2.2 Setting System Parameters	155
2.2.1 Specifying Masses	155
2.2.2 Specifying Formulae and Formula Ranges	156
2.2.2.1 Search Formula	156
2.2.2.2 Formula Range	156
2.2.2.3 Search Mass	157
2.2.3 Spectrum Data Filters	157
2.2.3.1 Spectrum Scan Range	157
2.2.3.2 Intensity Cutoff	158
2.2.3.3 Mass Range	158
2.2.4 Match Error	158
2.2.5 Current Spectrum	158
2.2.6 Auto Search Parameters	159
2.2.7 Printers and Plotters	159
2.3 Starting, Stopping and Resetting MassCalc	160
2.4 Main Screen Display & Viewing Other System Settings	160
2.6 Getting Help	161
2.7 Getting Data into MassCalc (RS)	161
2.8 Plotting and Listing Data	162
2.8.1 Listing the Elements and Fragments (PP A)	162
2.8.2 Listing the Spectra (PP P)	163
2.8.3 Plotting (PL)	163
2.9 Calculations	165
2.9.1 Calculate Mass (CM)	165
2.9.2 Isotope Patterns (IP)	165
2.9.3 Elemental Analysis (EA)	166
2.10 Analysis of Spectra	167
2.10.1 Automatic Search (AS)	167
2.10.2 Find a Formula or Mass (FF)	168
2.10.3 Formula Search (FS)	168
2.10.4 Print Differences Table (PD)	168
3.0 System Requirements	168
4.0 Installation	169
5.0 Error Messages	169

## 1.0 Introduction

---

The interpretation of mass spectra typically involves a considerable amount of tedious arithmetic analysis with a calculator and table of atomic weights: processes which are readily automated. While data systems for mass spectrometers often include programs for the analysis of spectra they are often inaccessible and complex to use.

MassCalc is a program designed to assist with the interpretation of mass spectra. It is simple to use and runs on the readily available IBM-PC computer. It is not an attempt at automated spectra interpretation, but rather a tool to assist the interpretation process.

There are functions which automate the arithmetic processes of calculating the sums and differences of peaks and looking for common fragments. Whole files of spectra may be searched, and mass range and intensity filters may be applied. Other functions perform the relatively non-trivial tasks of finding possible formulae for a mass, and calculating isotopic patterns for a formula. The data may be plotted and listed in a variety of ways that make the best possible use of any video hardware that is present. The results of the analyses may be printed or plotted.

### 1.1 The Atom/Fragment Data File

---

MassCalc requires two data files: the atom/fragment file MASSCALC.DAT, and a spectrum file.

The atom data file is formatted as shown below. The first column contains the symbol for the atom or fragment. This column has a fixed width of 6 characters. The next column is a twenty character description of the atom/fragment, also a fixed length field. The name should be terminated with a period. Following the name field is a one character field which assigns the data to the Element, Fragment or Alt Fragment lists. The three values which this field may have are "E", "F" or "A" correspond to the three lists respectively.

The remaining fields are free format. The fourth specifies how many isotopes the atom has, and the rest of the fields are mass and percentage abundance pairs. The masses should be specified at the highest precision possible. For example the atom/fragment with the symbol "C" is called "Carbon", it is an element and has two isotopes: 12 of 98.892% abundance and 13 with 1.102% abundance.

The file must be terminated with a control/Z (^Z) at the end of the last line in the file.

An element or fragment may appear in more than one list. AutoSearches will use all the entries in the Fragment or Alt Fragment lists, but direct references to an element/fragment will use the first entry in the list. Although the masses used for fragments may be the average mass it is recommended that the lowest isotopic mass be used.

C	Carbon.	E	2	12.0	98.892	13.00335	1.102
H	Hydrogen.	E	2	1.007825	99.984	2.01400	0.0156
O	Oxygen.	E	3	15.99491	99.759	17.9992	0.204 16.9991 0.037
N	Nitrogen.	E	2	14.00307	99.635	15.00011	0.365

Cl	Chlorine.	E 2	34.96885	75.53	36.9658	24.47
Br	Bromine.	E 2	78.9183	50.54	80.9183	49.46
SiSt1	Silyl steroid.	F 1	90.15	100.0		
CH2	Homologous.	F 1	14.0	100.0		
CH3	Methyl.	F 1	15.0	100.0		
Ox	Ar-NO2, NO, sulphoxide.	F 1	16.0	100.0		
Ala	Alanine.	A 1	89.1	100.0		
Asp	Aspartic acid.	A 1	133.1	100.0		
Cys	Cysteine.	A 1	121.2	100.0		

## 1.2 The Spectrum Data File

Spectroscopic data is contained in an ascii text file. This file may be created and modified with any editor, or may be generated directly from a mass spectrometer data system. The spectrum data file might contain a GCMS run, a series of spectra from a probe run, or a collection of spectra from various sources all related to a particular problem. High and low resolution spectra may be mixed in one file. The resolution of the spectra is determined from the number of significant figures after the decimal point on the mass: 3 or more is regarded as high resolution. The *Show (SH)* command will list the statistics on the spectra in memory.

The file may contain up to 1000 spectra, and each spectrum may have up to 5000 mass/intensity observations. The total number of peaks in the file is limited by the amount of memory available on the system. Each mass intensity pair uses 12 bytes and each spectrum has an overhead of about 90 bytes. If the computer has 640k of memory then there is room for about 35,000 peaks. The memory usage statistics are listed by the *Show (SH)* command.

The data is entered in free format. The start of each spectrum is marked by a *!BeginSpectrum* statement. The remainder of the line in which the *!BeginSpectrum* occurs is regarded as the spectrum title. The following lines have one or more pairs of mass/intensity observations. The end of the spectrum is marked by an *!EndSpectrum* statement. An *!EndRun* statement marks the end of the file.

The spectrum file is read into memory using the *Read Spectrum (RS)* command. The file may have any name, but the extension ".MSD" is the default used by MassCalc (e.g MYCAMD.MSD)

```
!BeginSpectrum JM5 32.8 24Aug87 14:14:00 #40-44 DCI/NH3'
550 10 536 10 526 8 518 30 501 25 483 35 462 30 452 30 400 25
398 25 394 25 384 40 382 35 376 20 368 60 352 30 350 30

!EndSpectrum
!BeginSpectrum Tedanolide EI!HiRes, ex F.J. Schmitz
678.98101 193 309.17083 141 291.16005 129 221.15540 267 193.08820 186
185.09058 351 175.06028 133 170.07384 355 169.08665 1087 168.05680 93
167.07084 42 165.12851 228 165.09346 53 163.11288 59 163.07808 57

!EndSpectrum

!BeginSpectrum Tedanolide FAB
633.3075 100 593.3201 100
!EndSpectrum

!EndRun
```



### 1.3 Printer and Plotters

---

The screen listings and plots which are generated by MassCalc may be spooled to disk files for later printing or plotting, or inclusion in other documents. Spooling is turned on by using the *SV PR +* command. The *SV PR <filename>* command may be used to specify the filename for the printed output (if *PRN* is used for the filename output will be sent directly to the printer). The filename cannot be changed once a *SV PR +* command has been issued. All printed output is sent to this file until an *SV PR -* command is issued. Once opened the spool file is not closed until MassCalc exits back to DOS, thus printed output may be selectively saved in the file by toggling the *[PR]* parameter on and off. Any 80 column printer may be used.

The plotted output is saved in a series of files called *MASSPLOT.xxx*, where "xxx" is the plot number which starts at "001" and runs up to the number of plots done. There is one plot per file. The plots are saved only when the printer spooling is *ON*. The plot files contain ascii plotter commands in Hewlett-Packard Plot Language (HPGL).

The printer and plotter spool files are deleted if a *Reset System (RE)* command is executed.

## 2.0 MassCalc Commands

---

MassCalc is controlled by a series of commands which are typed in response to the "MassCalc>" command prompt, and terminated by pressing the <Enter> key. The commands may be typed in upper or lower case letters. If part of the input required for a command is missing, MassCalc will prompt for the missing information. The following sections describe the commands and give examples of their use. At the end of each section is a "railroad" diagram which shows the possible syntax combinations for each command.

The previous twenty commands may be retrieved, by pressing the up/down arrow keys, and edited. The <home>, <end> and left/right arrow keys may be used to position the cursor. The <backspace> and <DEL> keys may be used to delete characters, and new characters are inserted at the cursor position. When editing is complete, the new command is executed by pressing the <ENTER> key.

### 2.1 MassCalc Command Structure

---

The MassCalc commands are all two letters long. Although commands such as *HE* may be typed as *HELP*, only the first two characters are significant. The commands are briefly summarised below in alphabetical order.

- *AS* - Automatic Search of spectra for fragments or possible formulae
- *CM* - Calculate the mass and % composition of a formula
- *EA* - Calculate a formula from an elemental analysis
- *EX* - Exit from the program. *QUIT* is equivalent
- *FF* - Find a formula or mass in spectrum list or differences
- *FS* - Molecular formulae search for a given mass or peak
- *IP* - Prints the isotope pattern for a formula
- *PD* - Print a table of mass differences for all peaks in a spectrum
- *PL* - Plot - plot spectra in various forms
- *PP* - Print parameters - element and spectral mass/int values
- *RE* - Reset the program to initial state i.e. start again
- *RS* - Read a file of spectra from disk

- *SH* - Show spectrum stats, memory usage etc
- *SV* - Set values of the parameters

Each command is typed, possibly followed by one or more parameters, then executed by pressing the <ENTER> key.

## 2.2 Setting System Parameters

MassCalc has a number of "control parameters." These parameters either affect the function of the various command, or supply default values for formulae and masses. The *SV* command is used to set the values of the control parameters. The settings of these parameters is shown either on the main display at the MassCalc prompt or by using the *SH* command.

Usage : *SV* <parameter name> <one or more parameter values>

Listed below is a brief description of the parameters:

- *AD* - Auto Search Depth sets the number of fragment combinations in an AutoSearch
- *AM* - Auto Search Mode sets the type of autosearch to be done
- *CS* - Current Spectrum in the spectrum file, used by *PD*, *PP*, *PL* and <mass>
- *FR* - Formula Range is the number of each atom allowed in formula searches
- *IC* - Intensity Cutoff limits processing to peaks with above the threshold
- *ME* - Match Error used for comparing masses. May be fixed number or "Auto"
- *MR* - Mass Range limits listings, searches and plots to part of a spectrum
- *PR* - Printer sets the printer plotter save files on and off.
- *SF* - Search Formula is the formula used by *IP*, *FF*, *CM*
- *SM* - Search Mass is the mass used by *FS*
- *SR* - Spectrum Range used to limit spectra to be used in *AS*, *FF* and *PL*

<system parameter> — FR, SF, SM, CS, SR, ME, MR, IC, AM, AD or PR —

*SV* — <system parameter> — <value(s)> —

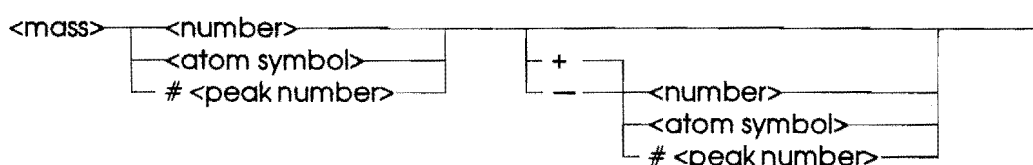
### 2.2.1 Specifying Masses

Anytime MassCalc requires a <mass> to be entered it may be specified in a number of ways. The mass can be entered as a number (eg "323.4567"), as an atom symbol (eg "Br" or "Ala") or as the index number of a peak in the Current Spectrum [*CS*] (eg "#5"). In addition the sum or difference of any two of these items may be calculated (eg "#5-32"), and the absolute value of the result used as the mass.

If the Match Error [*ME*] is set to "Auto" then the <mass> will have a precision associated with it. A number with 3 or more significant digits after decimal place, an atom symbol, and a peak number from a HiRes spectrum are considered high resolution, while numbers with less than 3 digits or peak numbers from a LoRes spectrum are considered LoRes. If a HiRes item is added/subtracted with a LoRes item the result is LoRes.

Examples :	386	<mass> = 386, LoRes
	386.13	<mass> = 386.13, LoRes
	386.1348	<mass> = 386.1348, HiRes
	Br	<mass> = 78.91830, HiRes

#17            <mass> = mass of peak 17 in Current Spectrum (CS)  
 386.1348+br    <mass> = 386.1348+78.91830 = 465.05310, HiRes  
 #17-23        <mass>= mass of peak 17 minus 23, LoRes  
 #1-#2         <mass> = peak 1 minus peak 2 of Current Spectrum

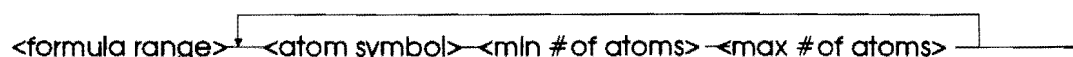


### 2.2.2 Specifying Formulae and Formula Ranges

A number of commands require either a <formula> or a <formula range>. A <formula> is standard chemical formula. The formula is represented in the usual manner of an atom symbol, followed by a number. For example C<sub>9</sub>H<sub>9</sub>Br<sub>3</sub>O<sub>2</sub> would be typed C9H9BR3O2. The atom symbol may be entered as either upper or lower case, and embedded spaces are not significant, thus c9h9Br3o2 and c9 H9 br3o2 are also valid. If an atom symbol appears more than once in a formula the entries for the atom are summed, e.g. acetic acid could be entered c1h3c1o2h1 and would be summed to C2 H4 O2.



A range specifies the upper and lower limits of the number of atoms/fragments of each type that may be used in a search. The atom symbol is typed, followed by two numbers: the lower and upper bounds. Once again the case of the atom symbol and embedded spaces are not important. For example C5 7 H5 20Br3 3o0 4.



#### 2.2.2.1 Search Formula

The Search Formula [SF] is the <formula> which is used by FF, CM and IP if a molecular formula is not explicitly specified in the command line. If a formula is entered on the command line of FF, CM or IP that formula becomes the new Search Formula.

Usage : SV SF <formula> <ENTER>

Examples : SV SF c9h9br3o2  
           : SV SF Br3  
           : SF C1H3 C1H2 C1O1 H1

#### 2.2.2.2 Formula Range

The Formula Range [FR] defines the range of numbers of each atom type that are permitted in a molecular formula search. The two commands which use [FR] are AS, option "S", and FS. If a formula range is not entered on the FS or AS command line the current setting of [FR] is used. If a formula range is entered on the command line then it becomes the new Formula Range.

Usage : SV FR <formula range>

Examples : SV FR c7 11h6 12 o0 3 Br 3 3  
 : SV FR c3 3 h12 13

### 2.2.2.3 Search Mass

[SM] sets the *Search Mass*. This is the value which is used by the FS command if a <mass> is not explicitly specified. The value of this parameter may be set with a number, set to the mass of a peak in the *Current Spectrum [CS]*, or set to the mass of the *Search Formula [SF]*. See the section entitled "Specifying Masses" for more information on the options on setting a mass. The precision to which the mass is specified determines the tolerance to be used for matching when the Match Error [ME] is set to "Auto". "HiRes" or "LoRes" is displayed alongside the [SM] value

Usage : SV SM <mass> or \*

Examples : SV SM 324.9832 - sets (SM) to a number, HiRes  
 SV SM #23 - set to mass of pk 23 in (CS)  
 SV SM \* - set to mass of Search Formula (SF)  
 SV SM #23-br - set to mass of pk 23 minus mass of Br  
 SV SM #4-#15 - set to mass of pk 4 minus pk 17

### 2.2.3 Spectrum Data Filters

The spectroscopic data seen by the various functions of MassCalc may be controlled with the three data "Filters". The data to be searched, plotted or listed may be limited to a range of masses, intensities and spectrum number. The following sections describe these three filters.

The peak index numbers which are listed by some functions are always the same regardless of the settings of the filters.

#### 2.2.3.1 Spectrum Scan Range

*Spectrum Scan Range [SR]* filter limits the spectra which will be processed to those which lie between and include the lower and upper limits of spectrum number. The FF, AS and PL 3D commands are affected.

The lower limit must be less than or equal to the upper limit. If the lower and upper limits are the same only one spectrum will be considered. Note that the PL 3D requires that more than one spectrum be plotted. If an asterisk "\*" is entered instead of the limits both the upper and lower limits will be set to the *Current Spectrum [CS]*

Usage : SV SR <lower limit > <upper limit > <ENTER>

Examples : SV SR 1 13 - set range from 1 to 13  
 SV SR 6 6 - set to use spectrum 6  
 SV SR \* - set lower & upper to Current Spectrum

### 2.2.3.2 Intensity Cutoff

The *Intensity Cutoff* [IC] filter limits the data which will be processed by *FF*, *AS*, *PL*, *PD*, *PP* commands. Only those peaks whose intensities are above [IC] will be accepted. The cutoff is expressed as a percentage (ie 0-100%) of the base peak for that spectrum.

Usage : SV IC <cutoff %>

Examples : SV IC 0.001  
SV IC 20

### 2.2.3.3 Mass Range

The *Mass Range* [MR] filter limits the data which will be processed by the *FF*, *AS*, *PL*, *PD* and *PP* commands. Only those peaks whose masses lie between and include the lower and upper limits will be accepted.

The lower limit must be less than the upper limit.

Usage : SV MR <lower limit> <upper limit>

Examples : SV MR 50 650  
SV MR 50.1 50.95

### 2.2.4 Match Error

The *Match Error* [ME] is used by MassCalc when comparing two masses for equality. The only exception is in the "*PP A=<mass>*" command where the comparison is of unit masses. If [ME] is set to a number then the match must be to that many mmu. If [ME] is set to "Auto" the matching tolerance will depend on the precision of the data. If the data have 3 or more significant figures after the decimal point then they are deemed to be high resolution data and the comparison will be required to match to within 2.0 mmu. Low resolution data must match within 500 mmu. Where "HiRes" data is being compared with "LoRes" data the matching is done at the LoRes tolerance.

When a file of spectra is read into memory the resolution of the data is determined for each spectrum. The *SH* command lists the spectra in memory with their resolution attribute. Masses derived from formulae are regarded as HiRes, while those entered as numbers may be HiRes or LoRes depending on the number of significant figures

Usage : SV ME <number> or Auto

Examples : SV ME 2 - set Match Error to 2 mmu  
SV ME A - set (ME) to "auto" mode

### 2.2.5 Current Spectrum

The *Current Spectrum* [CS] is the spectrum which is used as the default for the *PD*, *PL* and *PP* commands when a spectrum number is not explicitly specified. It is also used if a <mass> is specified using the #<peak number> construct where the mass is derived from the value of a peak in a spectrum. Note that the [CS] is NOT used by the *FF* and *AS* commands.

Usage : SV CS <spectrum number>

Example : SV CS 5 - set (CS) to spectrum 5 in the file

### 2.2.6 Auto Search Parameters

[AM] sets the *Autosearch Mode*. The Autosearch attempts to find molecular formulae for all the peaks, and all the differences between the peaks for all the data that lies within the *Mass Range* [MR], *Intensity Cutoff* [IC] and *Spectrum Scan Range* [SR] filters.

There are two types of Autosearch. The first is an extension of FS and performs a standard formula search on the data. The second type uses the two fragment lists to try to find likely fragments for the peaks or the differences between the peaks. There are two fragment lists. One has general organic chemistry fragments and the other can be set up for specialised applications such as sterols or amino acids. Either or both lists may be used in this second type of Autosearch. Varying the Autosearch Depth [AD] allows combinations of the fragments list to be tried as well.

Warning: if the filters are set too broadly the Autosearch may take a long time and produce a lot of not very useful solutions.

The four options for [AM] are:-

- "S" - formula search mode.
- "F" - try the Fragment List against the peaks and their differences.
- "A" - try the Alternate Fragment List.
- "B" - use both the Alt and the Fragment Lists.

Usage : SV AMS or F or A or B

[AM] sets the *Autosearch Mode*. The Autosearch attempts to find molecular formulae for all the peaks, and all the differences between the peaks, for all the data that lie within the *Mass Range* [MR], *Intensity Cutoff* [IC] and *Spectrum Scan Range* [SR] filters.

The *Autosearch Depth* [AD] applies to auto-searches using the Fragment or Alt Fragment lists. [AD] sets the number of combinations of fragments that may be tried against the peaks and their differences. A value of 1 means that each fragment in the list is tried against each possible mass. A value of 2 means that each fragment and each combination of two fragments is tried, and so on. Values for [AD] of up to 10 are legal, but values over 2 may result in searches which take a very long time and produce a large number of unhelpful solutions.

Usage : SV AD <depth>

Example : SV AD 1

### 2.2.7 Printers and Plotters

[PR] is the printer option. If the printer mode is ON the output from the various commands is saved in a disk file for later printing or plotting. The printer option is turned on and off with the SV PR command. Once ON all output is saved until the next OFF command. The last page of the SH command output shows the status of the printer and plotter files.

The text output from a session is saved in a file, which can be edited using the system editor, or printed using the DOS print command. The name of the file is set using the *SV PR* <filename> command, which can only be issued before the first *SV PR +*. If the filename is set to "PRN" the output will be sent directly to the printer. The default name for this file is "MASSLIST.LST." Since this file will be overwritten (ie destroyed) by subsequent MassCalc sessions you will need to change its name or copy it to another disk if you wish to keep it for future reference.

Plotted output is saved in a series of files called MASSPLOT.000 to MASSPLOT.nnn, where nnn is the number of plots you have done. There is one plot per file. The *IP* and *PL* commands save their output in this way. The file contains Hewlett Packard (HPGL) plot commands

Usage : *SV PR +* or -

Examples : *SV PR +* - printer/plotter spooling on  
*SV PR -* - printer/plotter spooling off

## 2.3 Starting, Stopping and Resetting MassCalc

---

MassCalc is started by typing "MASSCALC" at the DOS command prompt. The MassCalc program must either be in the current directory or in a directory pointed to by the current PATH (see the Installation section later in this manual).

The *EXIT* and *QUIT* commands finish a MassCalc session and return to DOS. The current settings of the various parameters such as the filters and MIM masses are saved in a file called MASSCALC.ENV in the current directory. These values will be restored next time MassCalc is run.

The *Reset (RE)* command resets MassCalc to its initial state. If a spectrum file is in memory it is cleared. The control parameters such as the filters and the MIM masses are restored to their initial values from the environment file. Any printer or plotter spool files which have been created during the session are deleted.

## 2.4 Main Display Screen and Viewing Other System Settings

---

When MassCalc is waiting for a command the following page is displayed on the screen. It shows the current values of the various control parameters.

The *Show (SH)* command displays a variety of information about the system and the spectrum which is loaded.

The first set of pages lists the data relating to the file of spectra currently in memory. The *Mass Range [MR]*, *Intensity Cutoff [IC]* and *Spectrum Range [SR]* filters have no effect on this listing. The meanings of the column headings are as follows:-

- *SpNo* - Spectrum Number
- *Res* - Resolution. Two or less figures after the decimal point is LoRes.
- *Pks* - Number of peaks in the spectrum.
- *BgNo* - Number of the background spectrum to be subtracted from here.
- *TIC* - Total Ion Current, ie the sum of all the intensities.
- *BasePk* - Intensity of the highest peak.
- *Title* - The title for this spectrum.

The final page gives various file and memory usage statistics.

SpectrumFile			
Run Name	:	MYCAMD.MSD	
Spectra In Run	:	8	Data Points : 302

Formula			
Formula Range (FR)	:	C5-11 H5-20 O0-4 Br3-3	
Search Formula (SF)	:	C9 H9 Br3 O2	Iso Mass : 0.00000
Search Mass (SM)	:	465.05310 HiRes	

Filter			
Current Spectrum (CS)	:	No.1 (LoRes) Mycalamide A DEI	
Spectrum Range (SR)	:	1-8	
Match Err (ME)	:	Auto	
Mass Range (MR)	:	50-650	Autosearch Mode : Srch Range
Int Cutoff (%) (IC)	:	0.5000	AutoSearch Depth : 1

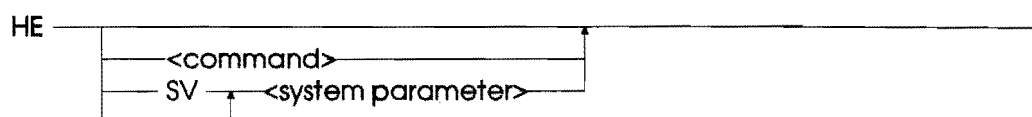
MIM Masses (MI)	
none defined	

Commands : HELP RS, FF, FS, PD, AS, CM, IP, EA, PP, PL, SH, SV, RE, EX  
MassCalc>

## 2.6 Getting Help

MassCalc includes an extensive "help" facility. Typing "HELP" at the MassCalc prompt will list the commands and a brief description of each one. Typing "HELP" then the name of a command or system parameter will list the detailed description.

Examples : HE  
          HE IP  
          HE SV MR  
          HE MR



## 2.7 Getting Data into MassCalc (RS)


The *Read Spectrum (RS)* command reads a spectrum file from disk into memory. If a spectrum file is already in memory it is replaced by the new one. The original file remains unchanged on disk. The RS command must be followed by a DOS filename. The filename may be a full pathname specifying disk and directory in the usual format: [*<dev>*:] [*<path>*] *<filename>* [*<ext>*]. If no extension, *<ext>*, is specified .MSD is assumed. If a no



filename is used, the names of the files in the current directory with a ".MSD" extension are listed.

Usage : RS <path/filename>

Examples : RS MYCAMD - read the file MYCAMD.MSD  
 RS MYCAMD.DAT - read the file MYCAMD.DAT  
 RS a:mycamd - read the file MYCAMD.MSD from drive A:  
 RS \data\mycamd - read from the directory \DATA

RS —————> 

## 2.8 Plotting and Listing Data

All or parts of the atom/fragment data may listed, and the data in the spectrum file can be either listed as mass/intensity pairs or plotted.

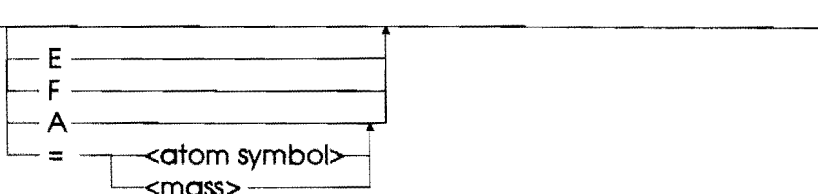
There are four ways of plotting the data. The first three summarise data from all the spectra with the limits of the *Spectrum Scan Range [SR]* filter. The spectra may be plotted as Total Ion Current (TIC) vs spectrum number, as selected ions (Multiple Ion Monitoring) vs spectrum number, or as a three dimensional projection of mass vs intensity vs spectrum number. Finally the *Current Spectrum [CS]* may be plotted as mass vs intensity. The *Current Spectrum* can also be plotted with another spectrum for comparison. The mass, intensity and spectrum number filters apply to the data plotted or listed.

### 2.8.1 Listing the Elements and Fragments (PP A)

The entire atom/fragment list may be displayed, or only the data for a particular atom, group of atoms/fragments, or a particular mass. Listing may be interrupted by striking any key while data is being written on the screen, or by typing "q" at the prompt at the bottom of the page. No filters apply and matching of the mass is to the nearest a.m.u.

Usage : PP A (E) or (F) or (A) or (= <mass> or <atom symbol>)

Examples : PP A - list entire atom table  
 PP A E - list elements only  
 PP A F - list fragment table  
 PP A A - list alternate fragment list  
 PP A = 32 - list all entries with mass of 32  
 PP A=br - list data for bromine

PP —————> 

### 2.8.2 Listing the Spectra (PP P)

The *PP P* command lists the mass/intensity data of the *Current Spectrum [CS]* a page at a time, with the highest mass first. Listing can be terminated by striking any key while data is being written on the screen, or by typing "q" at the prompt at the bottom of the page. The *Mass Range [MR]* and *Intensity Cutoff [IC]* filters control which ions are listed.

Usage : PP P (<spectrum number>)

Examples : PP P - lists the current spectrum  
PP P 5 - lists spectrum 5

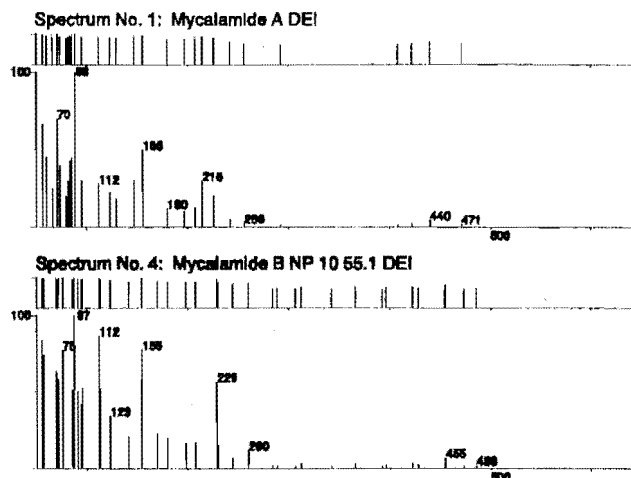
PP ——— P ————  
                  └───> <spectrum number> ───┘

### 2.8.3 Plotting (PL)

If the computer has a graphics capability then this function offers four methods of plotting the data. The Hercules, CGA, EGA and VGA adapters are supported by MassCalc. The Hercules and CGA adapter provide monochrome plots while the EGA and VGA produce 16 colour plots. If the printer option (the *SV PR+* command) is on, then the plots are saved in separate plot files called MASSPLOT.001 thru MASSPLOT. The *SH* command lists the names of the plot files. These files contain plot commands for Hewlett Packard and compatible plotters (HPGL). The *HELP PL* command will describe the type of graphics hardware found on your computer.

PL ————  
          ┌── TI ───┐  
          ├── MI ───┤  
          ├── 3D ───┤  
          └── N ───┘  
          └── SP ───┘  
                  └──> <spectrum number> ───┘

The data for the *Current Spectrum [CS]* may be displayed as a mass vs intensity plot. For comparison purposes a second spectrum may be shown below the *Current Spectrum*. When two spectra are displayed the peaks which occur in both spectra are marked. On



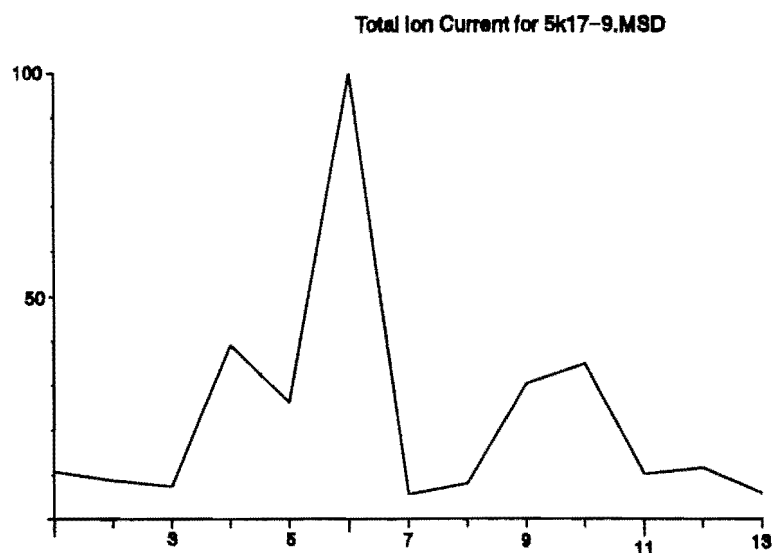
monochrome plots they are marked with an asterisk, while in colour the lines are drawn in cyan rather than red. The setting of the *Match Error [ME]* (see *SV ME* command) influences the peak matching. The *Mass Range [MR]* and *Intensity Cut-off [IC]* filters control which peaks are plotted. The peaks may be labelled with their mass by pointing the mouse cursor at the tip of the peak and clicking the left mouse button.

Usage : PL (SP (<n> ))

Examples : PL - plots current spectrum  
 PL SP - plots current spectrum  
 PL SP 4 - plots current spectrum and spectrum 4

The data can be summarised as a total ion plot, that is the sum of the intensities of all ions in each spectrum is plotted against spectrum number. The *Spectrum Range [SR]* is the only filter which has any effect.

Usage : PL TI

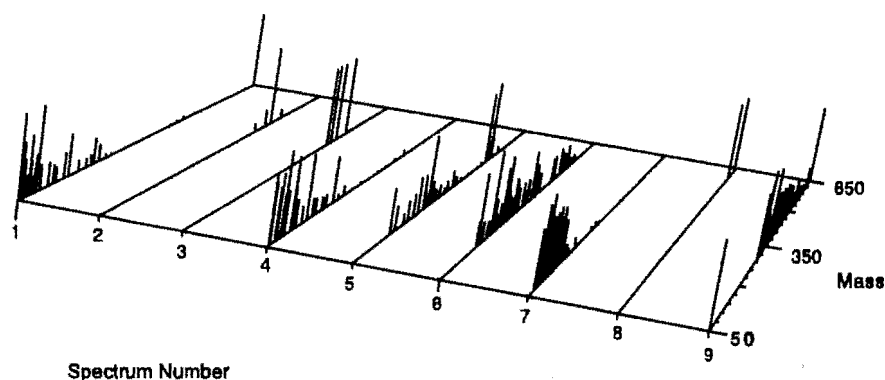


The intensity of selected ions may be plotted against spectrum number. Up to 10 masses may be selected for display using the *SV MI <n>...<m>* command. The *Spectrum Range [SR]* is the only filter which has any effect. The setting of the *Match Error [ME]* (see the *SV ME* command) influences the peak matching. The intensity of the ion plotted is the sum of all ions with fall within *[ME]* of the designated mass. This feature is not yet implemented.

Usage : PL MI

A 3-D projection of mass vs intensity vs spectrum number may be plotted. The *Mass Range [MR]*, *Intensity Cutoff [IC]* and *Spectrum Range [SR]* filters all control which peaks are plotted. The plot may take some time to draw. Drawing may be terminated by striking any key. Two views are drawn if a Hercules, EGA (256k) or VGA adapter is being used. The first plot, once drawn, is immediately moved to the "invisible" screen and a second view, rotated by 2.5 degrees drawn. When this is complete the display "rocks" between the two views. The display can be frozen by striking any key. The animated display is not drawn when printer/plotter spooling *[PR]* is ON. The peaks are scaled relative to the largest peak on the plot, unless the *PL 3D N* command is used to force to the base peak for each spectrum.

**File: mycamd.MSD**



## 2.9 Calculations

Three commands are provided to do commonly performed but tedious calculations. These are: calculate a mass from a formula, calculate the isotopic pattern for a formula and calculate the empirical or molecular formula from a micro-analysis.

### 2.9.1 Calculate Mass (CM)

The *Calculate Mass (CM)* command calculates the molecular mass and % composition of a molecular formula. The CM command may be followed, optionally, by a molecular formula. The current *Search Formula [SF]* is used, if no formula is entered. If a molecular formula is specified it will replace the current *Search Formula [SF]*.

Usage : CM (<molecular formula>)

Examples : CM  
CM C9H9Br3O2  
CM C30 H52 O12 N1

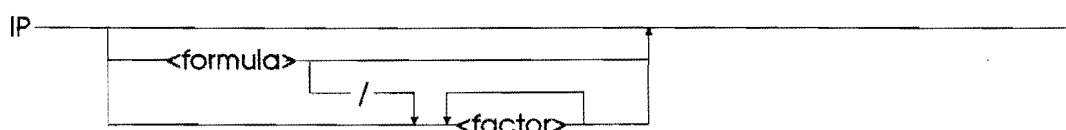


### 2.9.2 Isotope Patterns (IP)

The *Isotope Pattern (IP)* command displays the isotope pattern for a molecular formula. The *IP* command may be followed, optionally, by a molecular formula. The current *Search Formula [SF]* is used, if no formula is entered. If a molecular formula is specified it will replace the current *Search Formula [SF]*. If a set of numeric parameters is present then a series of plots are drawn with the M+1 ion overlayed on the M ion. If a formula is entered it must be terminated with a "/". For example "*IP cl2br3 / .5 1*" This command would draw the pattern for M then the pattern for an M with an M+1 overlayed at 0.5 the intensity of M, then a third plot with M+1 of equal size to the M ion. "*IP .5 1*" does the same using the *Search Formula*.

Usage : IP (<formula>) (<factors>)

Examples : IP - IP of current  
 IP C9H9Br3O2  
 IP C30 H52 O12 N1  
 IP .2 .5 1 1.5 2 - display series of overlayed M+1  
 IP c12br3 / .5 1 1.5 - display series of overlayed M+1

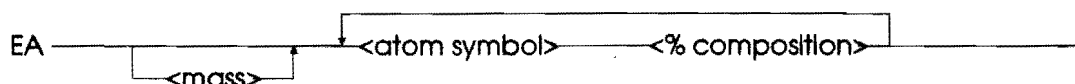


### 2.9.3 Elemental Analysis (EA)

The *Elemental Analysis (EA)* calculates the formula from a microanalysis. If the molecular weight is supplied then the result is the molecular formula, otherwise the empirical formula is shown. The calculation is based on the average molecular weight of the element. If an atom symbol from the fragment or alt fragment list is chosen the lowest isotope mass must be used, and an appropriate warning printed. The percentages of the items specified do not have to sum to 100%, but the sum must be less than 100%.

Usage : EA (<mass>) <elemental analysis>

Examples : EA 350.2234 c12.2 h3.3 o15.7 n5.5  
 EA c15.2 h2.3



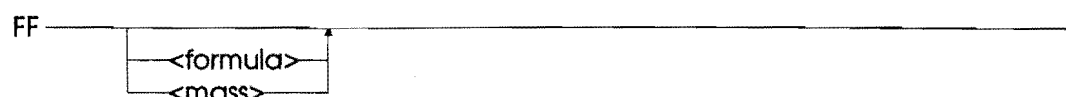
## 2.10 Analysis of Spectra

### 2.10.1 Automatic Search (AS)

*AutoSearch* takes all the peaks which lie within the bounds of the *Mass Range [MR]* and *Intensity Cutoff [IC]* filters and tries to find possible formulae for each peak and the differences between the peaks in the spectrum. Each spectrum within the bounds of the *Spectrum Scan Range [SR]* filter is processed in turn. The best results with this search are obtained by setting the filters as tight as possible. An all encompassing "blunderbuss" search will result in an overwhelming number of "hits."

The *AutoSearch Mode* parameter controls the type of search (see the *SV AM* command). If this is set to "Srch Range" a conventional formula search is performed using either the current *Formula Range [FR]*, or a formula range on the command line. This is equivalent to using the *FS* command on a range of peaks and spectra. The search may be interrupted by pressing any key.

Usage : AS (<formula range>)



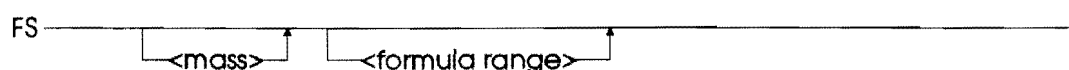
### 2.10.3 Formula Search (FS)

The *Formula Search (FS)* command searches for possible formulae for a given mass. The command line may specify either or both of the mass and formula range. If either the mass or formula range is omitted then the current settings of *Search Mass [SM]* or *Formula Range [FR]* are used respectively. The search time increases rapidly as the number of atom types increases. Searching may be interrupted by striking any key.

The matching of trial formulae is controlled by the setting of *Match Error [ME]*. If a number is set then the trial formula must have a mass within mmu of the target mass. When *[ME]* is set to "Auto" the match tolerance depends on the precision of the target mass. If the mass has 3 or more significant figures after the decimal or has been taken from a HiRes spectrum then the matching will be at HiRes (2.0mmu) otherwise the LoRes tolerance (500mmu) is used. The precision of the *[SM]* is shown beside it.

Usage : FS (<mass>) and/or (<mass> range)

Examples : FS - use current (SR) & (SM)  
 FS 323.9412 - HiRes using current (SR)  
 FS c20 20 h30 60 o0 5 - use current (SM)  
 FS 92 C3 3H10 15Si0 1 - LoRes using entered values



### 2.10.4 Print Differences Table (PD)

The *Print Differences (PD)* command generates a table of differences for all the masses in the Current Spectrum (see the *SV CS* command). There are no parameters for this command. The *Mass Range [MR]* and *Intensity Cutoff [IC]* filters control which data is listed.

Usage : PD

## 3.0 System Requirements

MassCalc runs under MS-DOS or PC-DOS (V2.00 or later) on any IBMPC or compatible.

Requirements:-

- IBM XT, AT, PS/2 or compatible.
- at least 256Kb of memory.
- VGA/EGA (at least 128Kb), CGA or Hercules Graphics board (optional).
- Hewlett-Packard HPGL compatible plotter (optional).
- 8087 Math Co-processor (optional).

The presence of graphics hardware, and its type is detected automatically by MassCalc. If more than one graphics adapter is present then EGA is selected before CGA before Hercules. The preferred graphics board is an EGA with 256Kb of memory. If an EGA is used it must have at least 128Kb of memory. Use the *HELP PL* command to find out what type of graphics hardware is on your system.

If a Math Co-processor is present the program will use it automatically.

The graphics displays may be plotted on any plotter which uses Hewlett-Packard HPGL.

#### 4. Installation

---

MassCalc may be run from either floppy or hard disks.

If MassCalc is to be run on a floppy based system the files on the distribution disk should be copied onto a working disk. Starting with a bootable diskette in drive A: and a newly formatted diskette in drive B: the following sequence of commands should be used:

```
SYS B:
COPY A:COMMAND.COM B:
COPY A:MASSCALC.* B:
COPY A:*.MSD B:
```

MassCalc is installed on a hard disk by copying the files from the distribution diskette to any convenient directory. In the example below the files will be copied to a directory called "MASSCALC" on drive C:

```
MD \MASSCALC
CD \MASSCALC
COPY A:MASSCALC.*
COPY A:*.MSD
```

The PATH command should be modified to include the directory where the MassCalc files reside. In the example above the following path command could be included in the AUTOEXEC.BAT file.

```
PATH C:\MASSCALC
```

#### 5. Error Messages

---

- *"This computer is not equipped for graphics"* - Attempt to plot spectral data on a computer with no graphics hardware.
- *"Unexpected end of input in spectrum file"* - The program came to the end of the spectrum file being read, without encountering an !ENDRUN or !ENDSPECTRUM statement.
- *"Missing !ENDSPECTRUM in spectrum , found 'xxxx'"* - Encountered an !ENDRUN statement before reading an !ENDSPECTRUM statement.
- *"Error reading INTENSITY in spectrum, found 'xxxx'"* - Invalid data was encountered while trying to read an intensity value. The offending string is displayed.
- *"Error reading MASS in spectrum, found 'xxxx'"* - Invalid data was encountered while trying to read a mass value. The offending string is displayed.
- *"Insufficient memory - remainder of file ignored"* - The spectrum file contains more data than can be held in memory. The balance of the file is ignored.
- *"There is no data file loaded, use the RS command"* - Attempt to execute a command which requires a spectrum file to be loaded. Use the RS command to read a spectrum file.



- "Current Spectrum is not defined - use "SV CS"" - Attempt to execute a command which uses the value of the *Current Spectrum* when none has been set. Either use the SV CS command to set the *Current Spectrum*, or explicitly specify the spectrum number on the command you were trying to execute.
- "No formula currently defined - use "SV SF"" - Attempt to execute a command which uses the value of the *Search Formula* when none has been set. Either use the SV SF command to set the *Search Formula*, or explicitly specify the formula on the command you were trying to execute.
- "No atom ranges currently defined - use "SV SR atom ranges"" - Attempt to execute a command which uses the value of the *Search Range* when none has been set. Either use the SV SR command to set the *Search Range*, or explicitly specify the range on the command you were trying to execute.
- "No match error currently defined - use "SV ME <number> or auto"" - Attempt to execute a command which uses the value of the *Match Error* when none has been set. Use the SV ME command to set the *Match Error*.
- "No search mass currently defined - use "SV SM "" - Attempt to execute a command which uses the value of the *Search Mass* when none has been set. Either use the SV SM command to set the *Search Mass*, or explicitly specify the mass on the command you were trying to execute.
- "Invalid search formula" - The *Search Formula* you specified was in the wrong format.
- "Invalid search range" - The *Search Formula* you specified was in the wrong format.
- "Invalid search mass [0.00001 - 9999.999]" - The *Search Mass* must be greater than 0 and less than 10 000.
- "Invalid match error [0.00001 - 1000.0 or Auto]" - The *Match Error* you specify must be greater than 0 and less than or equal to 1000, or be "AUTO".
- "Invalid autosearch mode [F,A,B,S]" - "F", "A", "B", "S" are the only valid automatic search options.
- "Invalid autosearch depth [1 - 10]" - The automatic search depth must be from one to ten.
- "Invalid intensity cutoff [0 - 100%]" - The *Intensity Cutoff* must be a positive number less than or equal to 100.
- "Invalid spectrum number range [1 - ]" - There was no spectrum number corresponding to the number you specified. Use the SH command to see a list of the spectra in memory.
- "Invalid mass range [0 - 9999.999]" - The mass must be greater than 0 and less than 10 000.
- "Mass Range must be greater then zero" - You specified a zero or negative *Mass Range*
- "Invalid printer option [— or + or <filename>]" - The printer option must be "+", "-" or a DOS filename.
- "Invalid current spectrum number [1 - ]" - There was no spectrum number corresponding to the number you specified. Use the SH command to see a list of the spectra in memory.

- *"Invalid MIM mass [0.00001 - 9999.999]"* - The masses must be greater than 0 and less than 10 000.
- *"Invalid parameter name"* - The valid parameter names are "FR", "SF", "SM", "CS", "SR", "ME", "MR", "IC", "AM", "AD" and "PR".
- *"Invalid plot type [SP, TI, MI, 3D]"*
- *"More than one spectrum must be plotted, increase spectrum scan range [SR]"* - Attempt to use a Spectrum Scan Range [SR] that includes only one spectrum, when the command you executed requires two or more. Use the "SV SR" to increase the range.
- *"Mass range [MR] for plot must be greater than zero"* - The current value of the Mass Range is zero. Use the "SV MR" command to increase it.
- *"Invalid file name [d:\dir\filename.ext]"* - The filename you specified was not a valid DOS filename.

## Geometry and Temperature Factor Tables

---

The following are the atomic position, bond length, bond angle and temperature factor tables for the crystal structures described in this thesis.

The equivalent isotropic temperature factor in the atomic position tables ( $U$ ) is defined as one third of the trace orthogonalised  $U_{ij}$  tensor.

The anisotropic temperature factor exponent takes the form:

$$-2\pi^2(h^2a^{*2}U_{11} + k^2b^{*2}U_{22} + \dots + 2hka^*b^*U_{12})$$

### ***3,5',7',7'-Tetrabromo-1',4-dibutyl-4'-(dibromomethylene)spiro (furan-2(5H),6'-(3)oxabicyclo(3.2.0)heptane)-2',5-dione (39).***

---

#### ***Atom Coordinates ( $\times 10^3$ ) and temperature factors ( $\text{\AA}^2 \times 10$ )***

<i>atom</i>	<i>x/a</i>	<i>y/b</i>	<i>z/c</i>	<i>U</i>
Br(4)	-943( 2)	6700( 2)	7954( 1)	48(1)
Br(4')	4684( 2)	3655( 2)	7293( 1)	48(1)
Br(6a)	845( 2)	8662( 2)	6681( 1)	65(1)
Br(6b)	4820( 2)	8120( 2)	6643( 2)	60(1)
Br(6c)	2210( 2)	2819( 2)	8604( 2)	66(1)
Br(6d)	-1724( 2)	1367( 2)	7756( 2)	59(1)
O(1)	4610(11)	7172(10)	7982( 4)	42(5)
O(1')	-546(11)	3497(10)	6740( 4)	44(5)
O(2)	5893(12)	8705(12)	9034( 5)	53(6)
O(2')	-1179(11)	4895(11)	5860( 4)	56(6)
C(2)	4525(22)	8071(15)	8644( 7)	51(9)
C(3)	2628(17)	8003(14)	8714( 6)	40(8)
C(4)	1573(18)	7149(14)	8120( 6)	42(7)
C(5)	2791(13)	6598(14)	7599( 6)	29(6)
C(6)	2421(15)	7088(14)	6860( 6)	37(7)
C(7)	1940(21)	8773(17)	9373( 7)	60(9)
C(8)	1688(17)	7553(19)	9855( 7)	58(9)
C(9)	3401(26)	6937(19)	10177( 8)	82(12)
C(10)	2870(20)	5563(19)	10625( 8)	69(10)
C(2')	-123(15)	4612(14)	6317( 6)	35(7)
C(3')	1963(14)	5255(14)	6492( 6)	31(7)
C(4')	2555(14)	4717(13)	7201( 6)	28(6)
C(5')	871(13)	3543(13)	7290( 6)	33(7)

C(6')	556(19)	2716(14)	7797( 6)	47(8)
C(7')	2764(20)	4593(14)	5856( 6)	48(8)
C(8')	2116(17)	2726(15)	5540( 6)	45(8)
C(9')	3249(20)	2089(15)	4997( 7)	55(9)
C(10')	2644(19)	222(16)	4697( 7)	57(9)

**Bond Lengths (Å)**

Br(4) - C(4)	1.848(14)	Br(4') - C(4')	1.912(11)
Br(6a) - C(6)	1.890(11)	Br(6b) - C(6)	2.020(12)
Br(6c) - C(6')	1.846(13)	Br(6d) - C(6')	1.895(14)
O(1) - C(2)	1.400(16)	O(1) - C(5)	1.439(13)
O(1') - C(2')	1.361(14)	O(1') - C(5')	1.392(13)
O(2) - C(2)	1.189(18)	O(2') - C(2')	1.178(14)
C(2) - C(3)	1.446(21)	C(3) - C(4)	1.341(17)
C(3) - C(7)	1.534(18)	C(4) - C(5)	1.522(17)
C(5) - C(6)	1.559(17)	C(5) - C(4')	1.579(16)
C(6) - C(3')	1.522(16)	C(7) - C(8)	1.488(20)
C(8) - C(9)	1.527(23)	C(9) - C(10)	1.575(22)
C(2') - C(3')	1.556(15)	C(3') - C(4')	1.544(16)
C(3') - C(7')	1.516(17)	C(4') - C(5')	1.533(15)
C(5') - C(6')	1.318(16)	C(7') - C(8')	1.533(17)
C(8') - C(9')	1.527(18)	C(9') - C(10')	1.523(18)

**Bond Angles (deg.)**

C(2) - O(1) - C(5)	109.4(10)	C(2') - O(1') - C(5')	112.3( 9)
O(1) - C(2) - O(2)	119.8(13)	O(1) - C(2) - C(3)	108.2(12)
O(2) - C(2) - C(3)	132.0(13)	C(2) - C(3) - C(4)	109.6(11)
C(2) - C(3) - C(7)	124.9(11)	C(4) - C(3) - C(7)	125.5(12)
Br(4) - C(4) - C(3)	126.1(10)	Br(4) - C(4) - C(5)	125.2( 8)
C(3) - C(4) - C(5)	108.7(11)	O(1) - C(5) - C(4)	104.0( 9)
O(1) - C(5) - C(6)	115.2( 9)	O(1) - C(5) - C(4')	111.9( 8)
C(4) - C(5) - C(6)	117.6( 9)	C(4) - C(5) - C(4')	121.8( 9)
C(6) - C(5) - C(4')	86.4( 8)	Br(6a) - C(6) - Br(6b)	104.0( 5)
Br(6a) - C(6) - C(5)	120.7( 8)	Br(6a) - C(6) - C(3')	121.4( 8)
Br(6b) - C(6) - C(5)	108.0( 7)	Br(6b) - C(6) - C(3')	109.3( 7)
C(5) - C(6) - C(3')	92.7( 9)	C(3) - C(7) - C(8)	112.2(11)
C(7) - C(8) - C(9)	116.5(12)	C(8) - C(9) - C(10)	109.8(14)
O(1') - C(2') - O(2')	123.6(10)	O(1') - C(2') - C(3')	108.7( 9)
O(2') - C(2') - C(3')	127.4(11)	C(6) - C(3') - C(2')	112.8( 9)
C(6) - C(3') - C(4')	89.0( 8)	C(6) - C(3') - C(7')	122.3(10)
C(2') - C(3') - C(4')	103.7( 9)	C(2') - C(3') - C(7')	107.0(10)
C(4') - C(3') - C(7')	120.1( 9)	Br(4') - C(4') - C(5)	115.9( 7)
Br(4') - C(4') - C(3')	117.1( 8)	Br(4') - C(4') - C(5')	111.3( 7)

C(5) - C(4') - C(3')	91.1( 8)	C(5) - C(4') - C(5')	116.0( 9)
C(3') - C(4') - C(5')	103.5( 9)	O(1') - C(5') - C(4')	109.8( 9)
O(1') - C(5') - C(6')	118.7(10)	C(4') - C(5') - C(6')	131.4(11)
Br(6c) - C(6') - Br(6d)	114.7( 6)	Br(6c) - C(6') - C(5')	124.0(10)
Br(6d) - C(6') - C(5')	121.2(10)	C(3') - C(7') - C(8')	115.3(10)
C(7') - C(8') - C(9')	111.6(10)	C(8') - C(9') - C(10')	111.7(11)

**Anisotropic temperature factors ( $\text{\AA}^2 \times 10^3$ )**

Atom	$U_{11}$	$U_{22}$	$U_{33}$	$U_{23}$	$U_{13}$	$U_{12}$
Br(4)	37( 1)	45( 1)	62( 1)	7( 1)	15( 1)	10( 1)
Br(4')	37( 1)	49( 1)	58( 1)	1( 1)	6( 1)	15( 1)
Br(6a)	92( 1)	44( 1)	59( 1)	16( 1)	15( 1)	27( 1)
Br(6b)	68( 1)	55( 1)	57( 1)	1( 1)	28( 1)	-20( 1)
Br(6c)	65( 1)	76( 1)	58( 1)	29( 1)	0( 1)	1( 1)
Br(6d)	49( 1)	37( 1)	90( 1)	13( 1)	26( 1)	1( 1)
O(1)	44( 5)	43( 5)	40( 5)	-7( 4)	8( 4)	-2( 4)
O(1')	50( 5)	37( 5)	44( 5)	3( 4)	-8( 4)	2( 4)
O(2)	31( 5)	74( 7)	54( 6)	-21( 5)	1( 5)	-24( 5)
O(2')	42( 5)	73( 6)	52( 6)	16( 5)	-3( 4)	9( 5)
C(2)	75(11)	34( 7)	45( 9)	2( 6)	23( 8)	8( 7)
C(3)	54( 9)	30( 7)	37( 8)	5( 6)	16( 7)	8( 6)
C(4)	66( 9)	27( 6)	34( 7)	6( 5)	16( 7)	18( 6)
C(5)	8( 5)	41( 7)	38( 7)	2( 5)	11( 5)	-8( 5)
C(6)	29( 7)	32( 7)	50( 8)	3( 6)	16( 6)	-9( 5)
C(7)	75(10)	53( 9)	51( 9)	-1( 7)	27( 8)	23( 7)
C(8)	38( 8)	87(11)	50( 8)	7( 8)	29( 7)	6( 7)
C(9)	128(16)	56(10)	61(10)	12( 8)	5(10)	15(10)
C(10)	49( 9)	81(11)	78(11)	27( 9)	16( 8)	-12( 8)
C(2')	24( 7)	44( 7)	37( 7)	5( 6)	7( 5)	-1( 5)
C(3')	23( 6)	34( 7)	35( 7)	7( 5)	10( 5)	-5( 5)
C(4')	19( 6)	29( 6)	37( 7)	4( 5)	2( 5)	-1( 5)
C(5')	7( 6)	29( 6)	62( 8)	5( 6)	3( 6)	-1( 5)
C(6')	80(10)	22( 6)	40( 7)	-2( 5)	15( 7)	16( 6)
C(7')	77(10)	36( 7)	32( 7)	0( 6)	4( 7)	13( 7)
C(8')	35( 7)	58( 9)	43( 7)	1( 6)	19( 6)	2( 6)
C(9')	76(10)	46( 8)	42( 8)	-4( 6)	17( 7)	11( 7)
C(10')	64(10)	46( 8)	61( 9)	-1( 7)	22( 7)	16( 7)

**(Z,Z)-5,5'-(1,2-Dibromo-1,2-ethanediylidene)bis(4-bromo-3-butyl -  
2(5H)-furanone) (40).**

---

**Atom Coordinates ( $\times 10^3$ ) and temperature factors ( $\text{\AA}^2 \times 10^2$ )**

atom	x/a	y/b	z/c	U
Br(1)	9108( 2)	-824( 1)	7332( 1)	56(1)
Br(1')	7492( 2)	295( 1)	5026( 1)	57(1)
Br(3)	3280( 1)	2173( 1)	6700( 1)	48(1)
Br(3')	7404( 2)	3035( 1)	8073( 1)	55(1)
O(2)	5606( 9)	-667( 6)	8523( 4)	45(4)
O(2')	7796( 9)	3216( 6)	5128( 4)	51(4)
O(9)	3606(10)	-1140( 8)	9838( 5)	63(5)
O(9')	8007(12)	5389( 8)	4529( 6)	77(6)
C(1)	7139(13)	300( 8)	7046( 6)	37(6)
C(2)	5663(13)	218( 9)	7674( 6)	39(5)
C(3)	3980(13)	926( 9)	7690( 6)	39(5)
C(4)	2976(13)	589(10)	8536( 6)	43(6)
C(5)	1223(13)	1102(11)	8953( 7)	49(6)
C(6)	1339(15)	2013(11)	9729( 7)	58(7)
C(7)	2257(18)	3270(11)	9394( 8)	78(9)
C(8)	2353(22)	4123(14)	10195(10)	105(12)
C(9)	3962(14)	-482(10)	9076( 6)	44(6)
C(1')	7428(12)	1189( 9)	6131( 6)	37(5)
C(2')	7601(13)	2492(10)	6055( 6)	40(6)
C(3')	7567(12)	3478( 9)	6729( 6)	40(5)
C(4')	7651(14)	4710(10)	6264( 7)	51(6)
C(5')	7601(16)	6081(10)	6592( 9)	68(8)
C(6')	5778(20)	6745(11)	6635(10)	83(9)
C(7')	4445(22)	6115(14)	7404(10)	93(11)
C(8')	2656(18)	6838(14)	7463(10)	88(10)
C(9')	7819(15)	4567(10)	5232( 8)	56(7)

**Bond Lengths ( $\text{\AA}$ )**

Br(1) - C(1)	1.902( 9)	Br(1') - C(1')	1.884( 9)
Br(3) - C(3)	1.867( 9)	Br(3') - C(3')	1.870( 9)
O(2) - C(2)	1.388(10)	O(2) - C(9)	1.403(12)
O(2') - C(2')	1.398(11)	O(2') - C(9')	1.383(12)
O(9) - C(9)	1.186(12)	O(9') - C(9')	1.198(14)
C(1) - C(2)	1.334(14)	C(1) - C(1')	1.462(12)
C(2) - C(3)	1.445(14)	C(3) - C(4)	1.334(13)
C(4) - C(5)	1.485(14)	C(4) - C(9)	1.475(14)

C(5) - C(6)	1.526(15)	C(6) - C(7)	1.470(16)
C(7) - C(8)	1.512(18)	C(1') - C(2')	1.310(13)
C(2') - C(3')	1.452(13)	C(3') - C(4')	1.321(13)
C(4') - C(5')	1.503(15)	C(4') - C(9')	1.461(15)
C(5') - C(6')	1.520(19)	C(6') - C(7')	1.481(21)
C(7') - C(8')	1.521(22)		

**Bond Angles (deg.)**

C(2) - O(2) - C(9)	107.6( 7)	C(2') - O(2') - C(9')	107.9( 7)
Br(1) - C(1) - C(2)	118.9( 7)	Br(1) - C(1) - C(1')	114.7( 7)
C(2) - C(1) - C(1')	126.4( 9)	O(2) - C(2) - C(1)	119.5( 8)
O(2) - C(2) - C(3)	107.6( 8)	C(1) - C(2) - C(3)	132.9( 8)
Br(3) - C(3) - C(2)	126.1( 7)	Br(3) - C(3) - C(4)	123.7( 8)
C(2) - C(3) - C(4)	110.2( 8)	C(3) - C(4) - C(5)	131.9( 9)
C(3) - C(4) - C(9)	106.3( 9)	C(5) - C(4) - C(9)	121.8( 8)
C(4) - C(5) - C(6)	112.0( 8)	C(5) - C(6) - C(7)	115.8( 9)
C(6) - C(7) - C(8)	113.5(10)	O(2) - C(9) - O(9)	119.9( 9)
O(2) - C(9) - C(4)	108.0( 7)	O(9) - C(9) - C(4)	132.2(10)
Br(1') - C(1') - C(1)	113.7( 6)	Br(1') - C(1') - C(2')	121.4( 7)
C(1) - C(1') - C(2')	124.9( 8)	O(2') - C(2') - C(1')	118.2( 8)
O(2') - C(2') - C(3')	106.1( 8)	C(1') - C(2') - C(3')	135.6( 8)
Br(3') - C(3') - C(2')	123.8( 7)	Br(3') - C(3') - C(4')	125.4( 7)
C(2') - C(3') - C(4')	110.9( 8)	C(3') - C(4') - C(5')	133.4( 9)
C(3') - C(4') - C(9')	106.2( 9)	C(5') - C(4') - C(9')	120.4( 9)
C(4') - C(5') - C(6')	111.6(10)	C(5') - C(6') - C(7')	115.3(11)
C(6') - C(7') - C(8')	114.2(12)	O(2') - C(9') - O(9')	120.0(10)
O(2') - C(9') - C(4')	108.9( 8)	O(9') - C(9') - C(4')	131.1(10)

**Anisotropic temperature factors ( $\text{\AA}^2 \times 10^3$ )**

Atom	$U_{11}$	$U_{22}$	$U_{33}$	$U_{23}$	$U_{13}$	$U_{12}$
Br(1)	49( 1)	65( 1)	53( 1)	4( 1)	-8( 1)	12( 1)
Br(1')	69( 1)	64( 1)	39( 1)	-7( 1)	-5( 1)	-1( 1)
Br(3)	47( 1)	58( 1)	40( 1)	17( 1)	-8( 1)	4( 1)
Br(3')	62( 1)	60( 1)	43( 1)	-3( 1)	-16( 1)	5( 1)
O(2)	50( 5)	53( 4)	33( 3)	19( 3)	-5( 3)	9( 3)
O(2')	65( 5)	53( 4)	35( 3)	14( 3)	0( 3)	-6( 4)
O(9)	58( 5)	83( 5)	48( 4)	31( 4)	-1( 4)	-5( 4)
O(9')	101( 8)	65( 5)	64( 5)	30( 4)	-4( 5)	-9( 5)
C(1)	49( 7)	30( 5)	32( 5)	-2( 4)	-10( 4)	-2( 4)
C(2)	42( 6)	46( 5)	29( 5)	1( 4)	-2( 4)	1( 4)
C(3)	45( 6)	45( 5)	28( 4)	4( 4)	-8( 4)	6( 4)
C(4)	37( 6)	60( 6)	32( 5)	7( 4)	-6( 4)	-3( 5)

C(5)	32( 6)	73( 7)	42( 5)	12( 5)	4( 5)	-11( 5)
C(6)	42( 7)	85( 8)	46( 6)	6( 6)	8( 5)	9( 6)
C(7)	75(10)	86( 8)	74( 8)	-11( 6)	-2( 7)	-1( 7)
C(8)	118(14)	90(10)	107(11)	-34( 9)	-30(10)	6( 9)
C(9)	47( 7)	55( 6)	30( 5)	9( 5)	-2( 4)	0( 5)
C(1')	30( 6)	49( 5)	32( 5)	1( 4)	-3( 4)	5( 4)
C(2')	33( 6)	52( 6)	34( 5)	9( 4)	-1( 4)	5( 4)
C(3')	36( 6)	44( 5)	40( 5)	5( 4)	-7( 4)	-4( 4)
C(4')	39( 7)	50( 6)	64( 6)	8( 5)	-10( 5)	-4( 5)
C(5')	58( 8)	49( 6)	98( 9)	2( 6)	-8( 7)	0( 6)
C(6')	125(13)	40( 6)	85( 9)	-5( 6)	-17( 9)	3( 7)
C(7')	123(14)	78( 9)	78( 9)	0( 7)	-8( 9)	29( 9)
C(8')	75(11)	93(10)	96(10)	-9( 8)	0( 8)	22( 8)
C(9')	60( 8)	49( 6)	58( 6)	16( 5)	-7( 6)	-12( 5)

### ***Eudistomin K p-bromobenzoate (41g)***

---

#### ***Fractional Atom Coordinates ( $\times 10^4$ ) and temperature factors ( $\text{\AA}^2 \times 10^3$ )***

atom	x/a	y/b	z/c	U
BR(1)	7152( 1)	0( 0)	5796( 1)	32(1)
BR(2)	8414( 1)	2829( 1)	8612( 1)	32(1)
S(1)	15923( 3)	2551( 1)	5297( 3)	28(2)
O(1)	15212( 7)	1806( 4)	3196( 8)	25(5)
O(2)	13953( 8)	1079( 3)	7936( 8)	28(5)
N(1)	13866(10)	1760( 4)	3449( 9)	22(6)
N(2)	12135( 9)	575( 4)	5074(10)	22(6)
N(3)	13765( 9)	1740( 4)	6250(10)	23(6)
C(1)	13951(12)	1183( 5)	4277(12)	23(7)
C(3)	12893(12)	1672( 6)	2183(12)	28(7)
C(4)	11396(12)	1674( 6)	2347(12)	26(7)
C(4a)	11320(11)	1243( 5)	3396(11)	20(3)
C(4b)	10164(11)	998( 5)	3782(12)	21(3)
C(5)	8737(12)	1093( 5)	3337(13)	28(3)
C(6)	7864(13)	795( 5)	3933(13)	29(3)
C(7)	8418(12)	381( 5)	4965(12)	25(3)
C(8)	9802(12)	272( 5)	5425(12)	25(3)
C(8a)	10697(12)	587( 5)	4826(12)	24(3)
C(9a)	12492(12)	999( 6)	4199(12)	27(3)
C(10)	14710(11)	1373( 5)	5665(11)	19(6)
C(11)	16064(11)	1738( 5)	5879(12)	22(7)
C(13)	15783(15)	2400( 6)	3614(13)	39(8)
C(15)	13358(12)	1525( 5)	7270(12)	23(7)



C(16)	12119(11)	1848( 5)	7506(12)	24(7)
C(17)	11025(12)	2045( 5)	6574(13)	28(7)
C(18)	9869(13)	2319( 5)	6850(13)	27(7)
C(19)	9888(11)	2408( 5)	8140(12)	24(7)
C(20)	10988(11)	2195( 5)	9113(12)	22(7)
C(21)	12102(12)	1914( 5)	8802(13)	24(7)
BR(1')	12191( 1)	5959( 1)	10808( 1)	36(1)
BR(2')	12497( 1)	3181( 1)	14416( 1)	32(1)
S(1')	2853( 3)	3429( 1)	10530( 3)	25(2)
O(1')	2631( 7)	4187( 3)	8476( 8)	26(5)
O(2')	6207( 8)	4617( 4)	13490( 8)	29(5)
N(1')	4145( 9)	4160( 5)	8714(10)	28(6)
N(2')	6968(10)	5221( 4)	10462( 9)	25(6)
N(3')	5763(10)	4106( 5)	11581(10)	28(6)
C(1')	4611(11)	4698( 5)	9618(11)	22(7)
C(3')	4384(11)	4307( 5)	7427(12)	23(7)
C(4')	5925(12)	4275( 5)	7504(12)	25(7)
C(4a')	6703(12)	4650( 5)	8639(12)	24(3)
C(4b')	8046(12)	4901( 5)	8972(12)	23(3)
C(5')	9172(12)	4859( 5)	8420(13)	31(3)
C(6')	10398(13)	5174( 5)	8971(13)	28(3)
C(7')	10513(12)	5514( 5)	10109(12)	23(3)
C(8')	9452(12)	5554( 5)	10710(13)	25(3)
C(8a')	8206(12)	5254( 5)	10121(12)	23(3)
C(9a')	6073(12)	4855( 5)	9551(13)	25(3)
C(10')	4568(11)	4507( 5)	11001(11)	18(6)
C(11')	3218(11)	4227( 5)	11165(12)	22(7)
C(13')	2122(13)	3608( 6)	8832(13)	35(8)
C(15')	6550(11)	4236( 5)	12773(12)	24(7)
C(16')	7958(12)	3927( 5)	13120(12)	22(7)
C(17')	8579(14)	3837( 5)	14427(12)	30(8)
C(18')	9947(14)	3608( 5)	14846(14)	34(8)
C(19')	10620(15)	3469( 6)	13912(16)	42(9)
C(20')	10004(11)	3548( 5)	12599(13)	28(7)
C(21')	8650(12)	3786( 6)	12224(13)	32(8)
Cm	5838(15)	2481( 6)	71(15)	45(4)
Om	6042( 8)	3124( 4)	-234( 8)	32(2)

**Bond Lengths (Å)**

BR(1) - C(7)	1.911(13)	BR(2) - C(19)	1.905(12)
S(1) - C(11)	1.832(12)	S(1) - C(13)	1.816(14)
O(1) - N(1)	1.446(12)	O(1) - C(13)	1.413(15)
O(2) - C(15)	1.245(14)	N(1) - C(1)	1.506(15)
N(1) - C(3)	1.475(16)	N(2) - C(8a)	1.398(15)

N(2) - C(9a)	1.416(16)	N(3) - C(10)	1.488(15)
N(3) - C(15)	1.348(16)	C(1) - C(9a)	1.496(17)
C(1) - C(10)	1.552(17)	C(3) - C(4)	1.556(18)
C(4) - C(4a)	1.473(17)	C(4a) - C(4b)	1.427(16)
C(4a) - C(9a)	1.372(17)	C(4b) - C(5)	1.403(16)
C(4b) - C(8a)	1.418(17)	C(5) - C(6)	1.367(18)
C(6) - C(7)	1.418(18)	C(7) - C(8)	1.369(17)
C(8) - C(8a)	1.403(17)	C(10) - C(11)	1.528(16)
C(15) - C(16)	1.497(16)	C(16) - C(17)	1.352(17)
C(16) - C(21)	1.411(20)	C(17) - C(18)	1.395(18)
C(18) - C(19)	1.401(19)	C(19) - C(20)	1.390(17)
C(20) - C(21)	1.382(17)	BR(1') - C(7')	1.909(12)
BR(2') - C(19')	1.921(15)	S(1') - C(11')	1.831(11)
S(1') - C(13')	1.833(14)	O(1') - N(1')	1.475(12)
O(1') - C(13')	1.421(15)	O(2') - C(15')	1.229(15)
N(1') - C(1')	1.498(15)	N(1') - C(3')	1.503(17)
N(2') - C(8a')	1.383(15)	N(2') - C(9a')	1.388(15)
N(3') - C(10')	1.472(14)	N(3') - C(15')	1.356(16)
C(1') - C(9a')	1.523(17)	C(1') - C(10')	1.559(17)
C(3') - C(4')	1.527(16)	C(4') - C(4a')	1.503(17)
C(4a') - C(4b')	1.406(17)	C(4a') - C(9a')	1.371(18)
C(4b') - C(5')	1.408(18)	C(4b') - C(8a')	1.424(18)
C(5') - C(6')	1.392(17)	C(6') - C(7')	1.406(18)
C(7') - C(8')	1.383(18)	C(8') - C(8a')	1.402(16)
C(10') - C(11')	1.529(16)	C(15') - C(16')	1.514(16)
C(16') - C(17')	1.403(19)	C(16') - C(21')	1.360(19)
C(17') - C(18')	1.416(19)	C(18') - C(19')	1.381(22)
C(19') - C(20')	1.405(21)	C(20') - C(21')	1.408(17)
Cm - Om	1.430(15)		

**Bond Angles (deg.)**

C(11) - S(1) - C(13)	99.0( 6)	N(1) - O(1) - C(13)	108.8( 9)
O(1) - N(1) - C(1)	104.7( 8)	O(1) - N(1) - C(3)	105.2( 9)
C(1) - N(1) - C(3)	111.8( 9)	C(8a) - N(2) - C(9a)	106.5( 9)
C(10) - N(3) - C(15)	121.8( 9)	N(1) - C(1) - C(9a)	105.9( 9)
N(1) - C(1) - C(10)	107.6( 9)	C(9a) - C(1) - C(10)	110.8(10)
N(1) - C(3) - C(4)	108.9(10)	C(3) - C(4) - C(4a)	109.1(10)
C(4) - C(4a) - C(4b)	131.1(10)	C(4) - C(4a) - C(9a)	121.1(11)
C(4b) - C(4a) - C(9a)	107.8(10)	C(4a) - C(4b) - C(5)	132.9(11)
C(4a) - C(4b) - C(8a)	106.7(10)	C(5) - C(4b) - C(8a)	120.4(11)
C(4b) - C(5) - C(6)	119.4(12)	C(5) - C(6) - C(7)	119.3(12)
BR(1) - C(7) - C(6)	117.3( 9)	BR(1) - C(7) - C(8)	119.4( 9)
C(6) - C(7) - C(8)	123.2(12)	C(7) - C(8) - C(8a)	117.4(11)
N(2) - C(8a) - C(4b)	109.0(10)	N(2) - C(8a) - C(8)	130.7(11)

C(4b) - C(8a) - C(8)	120.3(11)	N(2) - C(9a) - C(1)	122.6(10)
N(2) - C(9a) - C(4a)	110.0(10)	C(1) - C(9a) - C(4a)	127.1(11)
N(3) - C(10) - C(1)	109.7( 9)	N(3) - C(10) - C(11)	107.9( 9)
C(1) - C(10) - C(11)	118.4(10)	S(1) - C(11) - C(10)	116.0( 8)
S(1) - C(13) - O(1)	113.5( 9)	O(2) - C(15) - N(3)	122.0(11)
O(2) - C(15) - C(16)	123.1(11)	N(3) - C(15) - C(16)	114.9(10)
C(15) - C(16) - C(17)	124.5(12)	C(15) - C(16) - C(21)	115.8(11)
C(17) - C(16) - C(21)	119.7(11)	C(16) - C(17) - C(18)	122.1(13)
C(17) - C(18) - C(19)	117.8(12)	BR(2) - C(19) - C(18)	121.0( 9)
BR(2) - C(19) - C(20)	118.0(10)	C(18) - C(19) - C(20)	121.0(11)
C(19) - C(20) - C(21)	119.4(12)	C(16) - C(21) - C(20)	119.9(11)
C(11') - S(1') - C(13')	100.0( 6)	N(1') - O(1') - C(13')	110.1( 8)
O(1') - N(1') - C(1')	102.7( 8)	O(1') - N(1') - C(3')	103.3( 8)
C(1') - N(1') - C(3')	110.4( 9)	C(8a') - N(2') - C(9a')	107.6(10)
C(10') - N(3') - C(15')	119.9(10)	N(1') - C(1') - C(9a')	106.3( 9)
N(1') - C(1') - C(10')	110.2( 9)	C(9a') - C(1') - C(10')	111.4(10)
N(1') - C(3') - C(4')	109.7(10)	C(3') - C(4') - C(4a')	109.2(10)
C(4') - C(4a') - C(4b')	132.3(12)	C(4') - C(4a') - C(9a')	121.1(11)
C(4b') - C(4a') - C(9a')	106.4(11)	C(4a') - C(4b') - C(5')	133.4(12)
C(4a') - C(4b') - C(8a')	108.1(11)	C(5') - C(4b') - C(8a')	118.5(11)
C(4b') - C(5') - C(6')	120.3(12)	C(5') - C(6') - C(7')	119.2(12)
BR(1') - C(7') - C(6')	117.9( 9)	BR(1') - C(7') - C(8')	119.3( 9)
C(6') - C(7') - C(8')	122.8(11)	C(7') - C(8') - C(8a')	117.5(12)
N(2') - C(8a') - C(4b')	107.2(10)	N(2') - C(8a') - C(8')	131.2(12)
C(4b') - C(8a') - C(8')	121.6(11)	N(2') - C(9a') - C(1')	123.1(11)
N(2') - C(9a') - C(4a')	110.7(11)	C(1') - C(9a') - C(4a')	126.2(11)
N(3') - C(10') - C(1')	109.8( 9)	N(3') - C(10') - C(11')	112.0( 9)
C(1') - C(10') - C(11')	117.0(10)	S(1') - C(11') - C(10')	114.5( 8)
S(1') - C(13') - O(1')	111.8( 9)	O(2') - C(15') - N(3')	122.9(10)
O(2') - C(15') - C(16')	121.5(11)	N(3') - C(15') - C(16')	115.3(10)
C(15') - C(16') - C(17')	117.1(11)	C(15') - C(16') - C(21')	122.1(12)
C(17') - C(16') - C(21')	120.6(11)	C(16') - C(17') - C(18')	120.9(12)
C(17') - C(18') - C(19')	117.0(13)	BR(2') - C(19') - C(18')	119.1(12)
BR(2') - C(19') - C(20')	118.1(11)	C(18') - C(19') - C(20')	122.8(13)
C(19') - C(20') - C(21')	118.5(12)	C(16') - C(21') - C(20')	120.3(12)

**Anisotropic temperature factors ( $\text{\AA}^2 \times 10^{-3}$ )**

Atom	$U_{11}$	$U_{22}$	$U_{33}$	$U_{23}$	$U_{13}$	$U_{12}$
BR(1)	31( 1)	32( 1)	32( 1)	2( 1)	15( 1)	-3( 1)
BR(2)	25( 1)	37( 1)	32( 1)	-5( 1)	11( 1)	4( 1)
S(1)	24( 2)	32( 2)	28( 2)	1( 1)	1( 2)	-1( 1)
O(1)	11( 4)	43( 5)	21( 5)	4( 4)	6( 4)	1( 3)
O(2)	30( 5)	30( 4)	25( 5)	4( 4)	13( 4)	5( 4)

N(1)	19( 5)	36( 6)	11( 6)	-1( 5)	4( 5)	-5( 4)
N(2)	22( 5)	22( 5)	22( 6)	6( 4)	3( 5)	9( 4)
N(3)	21( 6)	25( 5)	24( 6)	9( 4)	3( 5)	2( 4)
C(1)	24( 6)	21( 6)	23( 8)	3( 5)	7( 6)	-6( 5)
C(3)	33( 8)	36( 7)	13( 7)	1( 6)	4( 6)	-9( 6)
C(4)	33( 8)	34( 7)	10( 7)	-2( 5)	-2( 6)	-13( 6)
C(10)	18( 6)	22( 6)	16( 7)	3( 5)	7( 6)	8( 5)
C(11)	18( 6)	41( 7)	8( 7)	9( 6)	2( 5)	1( 5)
C(13)	61(10)	33( 7)	22( 8)	14( 6)	10( 8)	-0( 7)
C(15)	19( 6)	23( 6)	26( 8)	9( 5)	10( 6)	-4( 5)
C(16)	19( 7)	26( 6)	26( 8)	8( 5)	13( 6)	5( 5)
C(17)	32( 7)	29( 7)	23( 8)	2( 6)	9( 6)	9( 5)
C(18)	34( 7)	27( 6)	22( 8)	3( 5)	-6( 6)	4( 5)
C(19)	22( 7)	24( 6)	25( 8)	12( 5)	11( 6)	1( 5)
C(20)	24( 7)	26( 6)	16( 7)	-1( 5)	0( 6)	-0( 5)
C(21)	14( 7)	27( 7)	32( 9)	6( 6)	-7( 6)	-5( 5)
BR(1')	28( 1)	32( 1)	47( 1)	-3( 1)	10( 1)	-5( 1)
BR(2')	25( 1)	30( 1)	41( 1)	2( 1)	-1( 1)	4( 1)
S(1')	24( 2)	25( 2)	26( 2)	-0( 1)	4( 2)	-2( 1)
O(1')	14( 4)	28( 5)	36( 6)	5( 4)	2( 4)	4( 3)
O(2')	35( 5)	32( 5)	20( 5)	-7( 4)	6( 4)	0( 4)
N(1')	21( 6)	36( 6)	27( 7)	-14( 5)	9( 5)	-2( 4)
N(2')	29( 6)	29( 5)	17( 6)	-11( 4)	11( 5)	-4( 4)
N(3')	27( 6)	25( 5)	30( 7)	-1( 5)	2( 5)	7( 5)
C(1')	23( 7)	23( 6)	19( 7)	2( 5)	5( 6)	-0( 5)
C(3')	18( 6)	29( 6)	23( 8)	8( 5)	-0( 6)	2( 5)
C(4')	35( 7)	24( 7)	17( 7)	-3( 5)	-3( 6)	-0( 5)
C(10')	14( 6)	21( 6)	19( 7)	-6( 5)	-2( 5)	3( 5)
C(11')	24( 7)	23( 6)	19( 7)	-6( 5)	5( 6)	-1( 5)
C(13')	31( 8)	47( 8)	27( 8)	-2( 7)	12( 7)	4( 6)
C(15')	23( 7)	25( 7)	25( 8)	-3( 5)	6( 6)	-5( 5)
C(16')	21( 6)	23( 6)	22( 8)	2( 5)	-2( 6)	1( 5)
C(17')	51( 9)	27( 6)	12( 7)	2( 5)	1( 7)	-2( 6)
C(18')	46( 8)	26( 7)	30( 9)	3( 6)	1( 7)	0( 6)
C(19')	55(10)	21( 7)	51(11)	-7( 7)	11( 9)	1( 6)
C(20')	19( 7)	38( 7)	27( 8)	-4( 6)	12( 6)	7( 5)
C(21')	22( 7)	59( 9)	16( 7)	-3( 6)	9( 6)	11( 6)

**Discorhabdin C (45).****Atom Coordinates ( $\times 10^3$ ) and temperature factors ( $\text{\AA}^2 \times 10^2$ )**

Atom	x/a	y/b	z/c	U
Br(2)	1145( 1)	3495( 1)	4501( 1)	19(1)

Br(4)	4505( 1)	6928( 1)	5899( 1)	18(1)
O(1)	-1381( 5)	2302( 4)	11779( 3)	41(3)
O(2)	344( 5)	2943( 4)	12565( 3)	41(3)
O(3)	3291( 4)	5885( 4)	4399( 3)	18(2)
O(4)	6665( 4)	4423( 4)	868( 3)	23(2)
O(5)	3179( 4)	4313( 4)	1342( 3)	24(2)
O(6)	6230( 4)	3866( 4)	7728( 3)	17(2)
O(11)	1299( 4)	-175( 3)	11241( 3)	17(2)
N(9)	534( 4)	2165( 4)	9864( 3)	14(2)
N(13)	3931( 4)	-1783( 4)	10373( 3)	11(2)
N(18)	4578( 4)	1575( 4)	7088( 3)	13(2)
C(1)	1361( 5)	2943( 5)	6628( 4)	12(2)
C(2)	1812( 6)	3831( 5)	5617( 4)	13(3)
C(3)	2818( 6)	5096( 5)	5320( 4)	12(3)
C(4)	3213( 5)	5313( 5)	6240( 4)	11(2)
C(5)	2759( 5)	4460( 4)	7244( 4)	10(2)
C(6)	1689( 5)	3195( 5)	7562( 4)	11(2)
C(7)	18( 6)	3494( 5)	8114( 4)	13(3)
C(8)	23( 6)	3470( 5)	9215( 4)	15(3)
C(10)	1611( 5)	1469( 5)	9453( 4)	12(2)
C(11)	2021( 6)	156( 5)	10322( 4)	12(2)
C(12)	3248( 6)	-568( 5)	9901( 4)	12(2)
C(14)	5030( 6)	-2050( 5)	9608( 4)	16(3)
C(15)	5080( 5)	-996( 5)	8631( 4)	12(2)
C(16)	5923( 6)	-689( 5)	7499( 4)	18(3)
C(17)	6073( 6)	867( 5)	6904( 4)	17(3)
C(19)	3605( 5)	1195( 5)	8047( 4)	12(2)
C(20)	2297( 5)	1941( 4)	8355( 4)	9(2)
C(21)	3972( 6)	-67( 5)	8822( 4)	13(3)
C(22)	-678( 6)	2204( 5)	12497( 4)	24(3)
C(23)	-1127( 6)	862( 6)	13511( 4)	25(3)
F(1)	-2485( 0)	201( 4)	13585( 3)	48(2)
F(2)	71( 4)	-36( 3)	13511( 3)	38(2)
F(3)	-1265( 4)	1058( 3)	14422( 2)	36(2)

**Bond Lengths (Å)**

Br(2) - C(2)	1.895( 6)	Br(4) - C(4)	1.898( 5)
O(1) - C(22)	1.226( 7)	O(2) - C(22)	1.220( 7)
O(3) - C(3)	1.219( 7)	O(11) - C(11)	1.214( 7)
N(9) - C(8)	1.438( 7)	N(9) - C(10)	1.327( 6)
N(13) - C(12)	1.369( 7)	N(13) - C(14)	1.371( 7)
N(18) - C(17)	1.478( 6)	N(18) - C(19)	1.333( 6)
C(1) - C(2)	1.338( 8)	C(1) - C(6)	1.489( 8)
C(2) - C(3)	1.476( 7)	C(3) - C(4)	1.474( 8)
C(4) - C(5)	1.320( 7)	C(5) - C(6)	1.501( 7)

C(6) - C(7)	1.552( 7)	C(6) - C(20)	1.517( 7)
C(7) - C(8)	1.512( 8)	C(10) - C(11)	1.535( 7)
C(10) - C(20)	1.407( 7)	C(11) - C(12)	1.431( 7)
C(12) - C(21)	1.391( 8)	C(14) - C(15)	1.379( 8)
C(15) - C(16)	1.498( 8)	C(15) - C(21)	1.397( 7)
C(16) - C(17)	1.525( 7)	C(19) - C(20)	1.429( 6)
C(19) - C(21)	1.429( 7)	C(22) - C(23)	1.557( 8)
C(23) - F(1)	1.327( 6)	C(23) - F(2)	1.367( 7)
C(23) - F(3)	1.332( 6)		

**Bond Angles (deg.)**

C(8) - N(9) - C(10)	121.9( 4)	C(12) - N(13) - C(14)	109.0( 4)
C(17) - N(18) - C(19)	121.8( 4)	C(2) - C(1) - C(6)	123.5( 5)
Br(2) - C(2) - C(1)	120.0( 4)	Br(2) - C(2) - C(3)	117.4( 4)
C(1) - C(2) - C(3)	122.6( 5)	O(3) - C(3) - C(2)	123.2( 5)
O(3) - C(3) - C(4)	122.9( 5)	C(2) - C(3) - C(4)	113.8( 5)
Br(4) - C(4) - C(3)	115.4( 4)	Br(4) - C(4) - C(5)	120.1( 4)
C(3) - C(4) - C(5)	124.5( 5)	C(4) - C(5) - C(6)	122.3( 5)
C(1) - C(6) - C(5)	112.9( 4)	C(1) - C(6) - C(7)	104.4( 4)
C(1) - C(6) - C(20)	112.3( 4)	C(5) - C(6) - C(7)	106.2( 4)
C(5) - C(6) - C(20)	112.3( 4)	C(7) - C(6) - C(20)	108.1( 4)
C(6) - C(7) - C(8)	112.3( 4)	N(9) - C(8) - C(7)	109.7( 4)
N(9) - C(10) - C(11)	111.6( 4)	N(9) - C(10) - C(20)	122.4( 5)
C(11) - C(10) - C(20)	126.1( 4)	O(11) - C(11) - C(10)	120.0( 5)
O(11) - C(11) - C(12)	127.6( 5)	C(10) - C(11) - C(12)	112.3( 4)
N(13) - C(12) - C(11)	131.9( 5)	N(13) - C(12) - C(21)	106.7( 4)
C(11) - C(12) - C(21)	121.4( 5)	N(13) - C(14) - C(15)	109.2( 4)
C(14) - C(15) - C(16)	136.4( 5)	C(14) - C(15) - C(21)	105.9( 5)
C(16) - C(15) - C(21)	117.6( 5)	C(15) - C(16) - C(17)	108.2( 4)
N(18) - C(17) - C(16)	114.5( 4)	N(18) - C(19) - C(20)	126.1( 5)
N(18) - C(19) - C(21)	114.8( 4)	C(20) - C(19) - C(21)	119.0( 5)
C(6) - C(20) - C(10)	121.0( 4)	C(6) - C(20) - C(19)	122.8( 5)
C(10) - C(20) - C(19)	116.2( 4)	C(12) - C(21) - C(15)	109.1( 5)
C(12) - C(21) - C(19)	124.4( 5)	C(15) - C(21) - C(19)	126.4( 5)
O(1) - C(22) - O(2)	132.4( 5)	O(1) - C(22) - C(23)	114.5( 5)
O(2) - C(22) - C(23)	113.0( 5)	C(22) - C(23) - F(1)	114.7( 5)
C(22) - C(23) - F(2)	109.3( 4)	C(22) - C(23) - F(3)	113.5( 5)
F(1) - C(23) - F(2)	106.5( 5)	F(1) - C(23) - F(3)	106.4( 4)
F(2) - C(23) - F(3)	105.9( 4)		

**Anisotropic temperature factors ( $\text{\AA}^2 \times 10^3$ )**

Atom	$U_{11}$	$U_{22}$	$U_{33}$	$U_{23}$	$U_{13}$	$U_{12}$
------	----------	----------	----------	----------	----------	----------

Br(2)	19( 1)	22( 1)	15( 1)	-7( 1)	-2( 1)	0( 1)
Br(4)	18( 1)	15( 1)	22( 1)	-5( 1)	3( 1)	-5( 1)
O(1)	44( 3)	57( 3)	21( 2)	-16( 2)	-6( 2)	22( 2)
O(2)	50( 3)	34( 3)	40( 3)	-6( 2)	9( 2)	-18( 2)
O(3)	24( 2)	13( 2)	16( 2)	-4( 1)	6( 2)	-4( 2)
O(4)	16( 2)	27( 2)	26( 2)	-11( 2)	-2( 2)	3( 2)
O(5)	22( 2)	27( 2)	22( 2)	-10( 2)	2( 2)	5( 2)
O(6)	20( 2)	17( 2)	15( 2)	-1( 2)	-1( 2)	-2( 2)
O(11)	16( 2)	21( 2)	13( 2)	-4( 1)	7( 1)	-1( 2)
N(9)	8( 2)	22( 2)	11( 2)	-6( 2)	4( 2)	1( 2)
N(13)	10( 2)	10( 2)	12( 2)	-1( 2)	4( 2)	-1( 2)
N(18)	9( 2)	18( 2)	12( 2)	-4( 2)	1( 2)	6( 2)
C(1)	5( 2)	13( 2)	18( 3)	-5( 2)	0( 2)	2( 2)
C(2)	8( 3)	15( 3)	17( 2)	-7( 2)	-2( 2)	8( 2)
C(3)	9( 3)	15( 3)	13( 2)	-5( 2)	3( 2)	5( 2)
C(4)	3( 2)	11( 2)	18( 3)	-6( 2)	6( 2)	0( 2)
C(5)	5( 2)	11( 2)	15( 2)	-6( 2)	3( 2)	3( 2)
C(6)	6( 2)	15( 3)	12( 2)	-4( 2)	3( 2)	1( 2)
C(7)	9( 3)	15( 3)	16( 2)	-5( 2)	4( 2)	6( 2)
C(8)	12( 3)	16( 3)	18( 3)	-3( 2)	4( 2)	5( 2)
C(10)	2( 2)	19( 3)	15( 2)	-8( 2)	2( 2)	0( 2)
C(11)	7( 2)	12( 2)	16( 2)	-4( 2)	0( 2)	-4( 2)
C(12)	13( 3)	12( 2)	11( 2)	-2( 2)	1( 2)	-4( 2)
C(14)	11( 3)	14( 3)	22( 3)	-8( 2)	0( 2)	3( 2)
C(15)	8( 3)	13( 2)	14( 2)	-5( 2)	0( 2)	3( 2)
C(16)	15( 3)	22( 3)	18( 3)	-11( 2)	0( 2)	8( 2)
C(17)	13( 3)	21( 3)	17( 3)	-2( 2)	8( 2)	6( 2)
C(19)	7( 2)	15( 3)	13( 2)	-8( 2)	2( 2)	-5( 2)
C(20)	6( 2)	8( 2)	13( 2)	-2( 2)	2( 2)	-2( 2)
C(21)	9( 3)	16( 3)	15( 2)	-7( 2)	0( 2)	-1( 2)
C(22)	18( 3)	28( 3)	25( 3)	-19( 2)	-2( 2)	8( 2)
C(23)	19( 3)	30( 3)	26( 3)	-18( 3)	-6( 2)	7( 3)
F(1)	35( 2)	44( 2)	65( 3)	-19( 2)	-9( 2)	-15( 2)
F(2)	37( 2)	31( 2)	47( 2)	-14( 2)	-2( 2)	9( 2)
F(3)	44( 2)	42( 2)	23( 2)	-14( 2)	4( 2)	1( 2)

## Publications

---

The following papers include work which has been described in this thesis, and are reproduced on the succeeding pages:

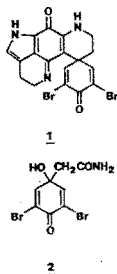
- Perry, N.B., Blunt, J.W., McCombs, J.D. and Munro, M.H.G., *Discorhabdin C, a Highly Cytotoxic Pigment from a Sponge of the Genus Latrunculia*, *J. Org. Chem.*, **51**, 5477 (1986).
- Blunt, J.W., Calder, V.L., Fenwick, G.D., Lake, R.J., McCombs, J.D., Munro, M.H.G. and Perry, N.B., *Reverse Phase Flash Chromatography: A Method for the Rapid Partitioning of Natural Product Extracts*, *J. Nat. Prod.*, **50**, 290 (1987).
- Lake, R.J., McCombs, J.D., Blunt, J.W., Munro, M.H.G. and Robinson, W.T., *Eudistomin K: Crystal Structure and Absolute Stereochemistry*, *Tetrahedron Lett.*, **29**, 4971 (1988).
- McCombs, J.D., Blunt, J.W., Chambers, M.V., Munro, M.H.G. and Robinson, W.T., *Novel 2(5H) Furanones from the Red Marine Alga Delisea elegans*, *Tetrahedron*, **44**, 1502 (1988).
- Blunt, J.W., McCombs, J.D., Munro, M.H.G. and Thomas, F.N., *Complete Assignment of the  $^{13}\text{C}$  and  $^1\text{H}$  NMR Spectra of Thyrsiferyl Acetate*, *Magn. Reson. in Chem.*, in press.



**Discorhabdin C, a Highly Cytotoxic Pigment from a Sponge of the Genus *Latrunculia***

**Summary:** The cytotoxic sponge pigment discorhabdin C (1) was shown, by a single-crystal X-ray diffraction study, to contain a new tetracyclic iminoquinone chromophore with a spiro 2,6-dibromocyclohexadienone.

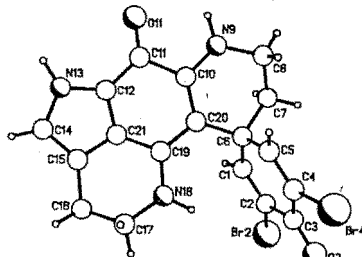
**Sir:** The strong cytotoxicity of extracts from various sponges of the genus *Latrunculia* du Bocage was detected in our wide-scale screening of New Zealand's marine invertebrates for antiviral and antitumor activity.<sup>1</sup> Bioassay-directed analysis of one such extract led to the isolation of a compound named discorhabdin C (1).<sup>2</sup> This compound, the major pigment of the red-brown sponge, is toxic toward L1210 tumor cells at very low levels (ED<sub>50</sub> < 100 ng/mL).



Percolation of MeOH/toluene (3:1) through the freeze-dried sponge led to an extract which was crudely partitioned by reverse-phase flash chromatography.<sup>3</sup> The cytotoxic fractions were combined and purified by two further stages of RPLC to give discorhabdin C (1), which was characterized as its hydrochloride: mp > 360 °C; [α]<sub>D</sub><sup>25</sup> 0°; IR (KBr) 3700–2500, 1675, 1585, 1540, 1325, 1020, 695 cm<sup>-1</sup>; UV (MeOH) 245 (ε 28500), 351 (10000), 545 nm (500); UV (MeOH/KOH) 337 (ε 13000), 481 nm (1500). Low-resolution FABMS gave a complex collection of ions around *m/z* 464, MH<sup>+</sup>, interpreted as a dibromo isotope pattern complicated by reduction in the FABMS matrix (as observed for quinones).<sup>4</sup> The molecular formula of the free base, C<sub>15</sub>H<sub>12</sub>Br<sub>2</sub>N<sub>2</sub>O<sub>2</sub>, was determined by high-resolution FABMS.<sup>5</sup> Discorhabdin C hydrochloride was slightly soluble in MeOH and H<sub>2</sub>O and soluble in Me<sub>2</sub>SO. The <sup>1</sup>H NMR spectra indicated 11 nonexchangeable and three exchangeable protons.<sup>6</sup> The <sup>13</sup>C NMR spectrum showed 16 carbon signals, two of which each represented two equivalent carbons, and an SFORD experiment confirmed the presence of 11 protons attached to carbons.<sup>7</sup>

The complex heterocyclic framework of discorhabdin C (1) was determined by a single-crystal X-ray diffraction study. Precession photography of a small crystal of the trifluoroacetate salt indicated a triclinic crystal system. Accurate cell constants, determined by a least-squares fit

of 23 high angle reflections, were *a* = 8.470 (2) Å, *b* = 10.562 (2) Å, *c* = 13.810 (2) Å, α = 67.84 (1)°, β = 78.53 (1)°, and γ = 88.38 (2)°, in space group *P*1. Two molecules per unit cell gave a calculated density of 1.87 g cm<sup>-3</sup>. All unique diffraction maxima with 2θ < 50° were collected, at 150 K, on a Nicolet R3m four-circle diffractometer using a variable speed ω scan technique and graphite monochromated Mo Kα radiation (0.7107 Å). A total of 3356 unique reflections were measured and, after correction for background, Lorentz, and polarization effects, the 2878 reflections with values of *R*<sub>int</sub><sup>2</sup> > 3σ were used for the structure solution and for the refinement of structural



**Figure 1.** Computer-generated perspective drawing of discorhabdin C.

parameters, by standard heavy atom methods.<sup>8</sup> The bromine positions were located from the Patterson synthesis and the remaining non-hydrogen atoms were located in subsequent Fourier calculations. The initial ambiguities in the scattering factor assignments were resolved with the aid of the spectroscopic data, and by analysis of the behavior of the temperature factors during the refinement calculations. Six water molecules were found in the unit cell. Hydrogen atoms were included in calculated positions. After applying an empirical absorption correction, a full least-squares refinement with anisotropic non-hydrogen atoms and isotropic hydrogens converged to a standard crystallographic *R* factor of 0.055.

Figure 1 is a computer-generated perspective drawing of protonated discorhabdin C. The chromophore is approximately planar, with the two six-membered heterocyclic rings in half-chair conformations: C7 above and C17 below the plane. The N9–C10 and N18–C19 bond lengths are both about 1.33 Å, demonstrating similar double-bond character and delocalization of the positive charge between these two nitrogens. The free base is arbitrarily represented as the 1,4-iminoquinone tautomer (1). The cyclohexadienone ring is planar, tilted at an angle of 76° to the plane of the chromophore. There is considerable strain at C6, the spiro ring junction, with bond angles ranging from 104° to 113° and the C6–C7 bond unusually long at 1.58 Å. Also, the cyclohexadienone ring is "pushed away" from N18, as the angle C3–C6–C20 is 23° larger than the

C3–C6–C7 angle. Conformational exchange must occur in solution since the NMR signals of C1 and C5 and of C2 and C4 are equivalent.

Discorhabdin C (1) has a new molecular skeleton, and we can find no previous reports of the pyrrolo[1,7]-phenanthroline skeleton of the iminoquinone chromophore. There is only one other report of metabolites (apart from sterols and fatty acids) from a *Latrunculia* species: the latrunculins are also toxins but are 2-thiazolidinone-bearing macrolides.<sup>9</sup> The 2,6-dibromocyclohexadienone portion of discorhabdin C (1) is clearly related to compound 2, isolated from another sponge as a product of tyrosine metabolism.<sup>10</sup> Discorhabdin C (1) could well be derived from some such tyrosine derivative (C1 to C8, N9) and from a tryptamine derivative (C10 to C21, N13, and N18).

Discorhabdin C (1) represents a new class of potential antitumor agents and is being tested further. Related compounds, isolated from another *Latrunculia* species, are also being studied.

**Acknowledgment.** This work was supported by the Harbor Branch Oceanographic Institution, Princeton, NJ, the Canterbury Medical Research Foundation, and the New Zealand University Grants Committee. Sponge identification was by Dr. Graham Fenwick, and bioassays were made by Vicky Calder, both of this Department. Low-resolution FABMS results were provided by Dr. Pat Holland, Soil Research Institute, Ruakura. High-resolution FABMS measurements were provided by J. C. Cook, Jr., University of Illinois. Low-temperature X-ray work was done in this Department with the help of Dr. Ward T. Robinson.

**Registry No.** 1, 105372-81-4; 1-HCl, 105372-82-5.

**Supplementary Material Available:** Tables of fractional coordinates, thermal parameters, bond distances and bond angles (5 pages). Ordering information is given on any current masthead page.

Nigel B. Perry, John W. Blunt\*  
John D. McCombs, Murray H. G. Munro\*  
Department of Chemistry  
University of Canterbury  
Private Bag, Christchurch, New Zealand  
Received July 23, 1986

- (1) Presented in part at the PAC CHEM 84 Congress, Honolulu, HI, Dec., 1984.
- (2) One characteristic of the family Latrunculiidae Topsent is the possession of discorhabd microcleres: Bergquist, P. R. *Sponges*; Hutchinson: London, 1978.
- (3) Blunt, J. W.; Calder, V. L.; Fenwick, G. D.; Lake, R. J.; McCombs, J. D.; Munro, M. H. G.; Perry, N. B., submitted for publication in *J. Nat. Prod.*
- (4) Cooper, R.; Unger, S. *J. Antibiot.* 1985, 38, 24.
- (5) MH<sup>+</sup>, *m/z* 461.9451, C<sub>15</sub>H<sub>12</sub>Br<sub>2</sub>N<sub>2</sub>O<sub>2</sub> requires 461.9452.
- (6) <sup>1</sup>H NMR (MeOH-*d*<sub>4</sub>, 300 MHz) δ 7.73 (s, 2 H, H1 + H5), 7.22 (s, 1 H, H14), 3.79 (t, *J* = 7 Hz, 2 H, H17), 3.73 (t, *J* = 6 Hz, 2 H, H8), 2.90 (t, *J* = 7 Hz, 2 H, H16), 2.12 (t, *J* = 6 Hz, 2 H, H7); <sup>1</sup>H NMR (Me<sub>2</sub>SO-*d*<sub>6</sub>, 80 MHz) δ 13.3 (s, 1 H, H13), 10.3 (s, 1 H, H9), 8.3 (s, 1 H, H18), 7.8 (s, 2 H, H1 + H5), 7.5 (s, 1 H, H14), 4.0–3.8 (m, 4 H, H8 + H17), 3.0 (t, *J* = 7 Hz, 2 H, H16), 2.1 (br s, 2 H, H7).
- (7) <sup>13</sup>C NMR (Me<sub>2</sub>SO-*d*<sub>6</sub>, 20 MHz) 171.5 (s, C3), 165.5 (s, C11), 153.3 (s), 151.9 (s), 151.4 (2 × d, C1 + C5), 127.8 (d, C14), 123.7 (s), 123.3 (s), 122.7 (2 × s, C2 + C4), 120.0 (s), 91.8 (s, C20), 44.8 (s, C6), 43.8 (t, C17), 38 (t, C8), 33.8 (t, C7), 18.2 (t, C16).
- (8) All calculations (including diagrams) were done on a Nova 4X computer by using SHELXTL: Sheldrick, G. M. "SHELXTL User Manual"; Nicolet XRD Corporation: Cupertino, CA, 1983; Revision 4.
- (9) Kashman, Y.; Growns, A.; Lidor, R.; Blumberg, D.; Carmely, S. *Tetrahedron* 1985, 41, 1905.
- (10) Tymiak, A. A.; Rinehart, K. L., Jr. *J. Am. Chem. Soc.* 1981, 103, 6763.

REVERSE PHASE FLASH CHROMATOGRAPHY: A METHOD FOR THE RAPID PARTITIONING OF NATURAL PRODUCT EXTRACTS<sup>1</sup>JOHN W. BLUNT,\* VICTORIA L. CALDER, GRAHAM D. FENWICK,  
ROBIN J. LAKE, JOHN D. MCCOMBS,  
MURRAY H.G. MUNRO,\* and NIGEL B. PERRY

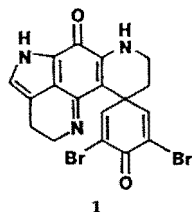
Department of Chemistry, University of Canterbury, Christchurch, New Zealand

The separation of the complex matrix of compounds in any natural product extract presents a daunting problem to the natural product chemist. Although a variety of approaches are available to reduce the complexity of the problem, probably the most widely used method is that of liquid/liquid partitioning. A number of partitioning schemes are possible that rely on combinations of solvents for the separation of different classes of compounds. This technique can be used readily on quite large samples with no requirement for expensive equipment. Pertit *et al.* give a good example of a complex but effective liquid/liquid partitioning scheme developed over several years for one particular extract (1).

The discovery of potent antiviral and antitumor activities in certain New Zealand marine invertebrates has necessitated the partitioning of natural product extracts on a scale ranging from a few grams to more than a kilogram. Partitioning of these extracts by liquid/liquid extraction and the subsequent assaying of the resultant fractions for antiviral and/or antitumor activities indicated that an active component was often distributed over several fractions of quite different polarities. The liquid/liquid partitioning technique was clearly not sufficiently selective with these extracts.

As the compounds of interest in these marine invertebrate extracts tended to be polar, an alternative approach based on the use of reverse-phase chromatographic adsorbents was explored. The overall aims were to have a system that

was fast, did not require expensive equipment, and yet would still be capable of partitioning the components of an extract with greater resolution than the usual liquid/liquid partitioning method. Shimizu has recently reviewed the problems presented by such extracts (2). He dismissed reverse-phase chromatography as an initial fractionation technique because of low sample capacity and the expense of commercial columns. However, the capacity of reverse-phase supports has been much underrated, and by the simple expedient of preparing reverse-phase supports (3) and using a low-pressure flash chromatography technique (4), both of these problems were overcome. The technique is illustrated by the isolation of the highly cytotoxic, water-soluble sponge pigment, discorhabdin C (1) (5).



## RESULTS AND DISCUSSION

The method that evolved is to coar the extract, containing compounds ranging from salts to hydrocarbons, on to a reverse-phase support. This can then be loaded, as either an aqueous slurry or a powder, onto a flash chromatography column that has been slurry-packed with the same support. Elution with H<sub>2</sub>O, followed by a steep, stepped gradient

through MeOH to CH<sub>2</sub>Cl<sub>2</sub> generally gives very satisfactory partitioning of crude extracts. The recovery of material is usually very good.

In the example chosen, a strongly cytotoxic extract prepared from a *La-trunculia* species of sponge was partitioned. The results of the partitioning are shown in Table 1. Analysis of the

a form ideally suited for further purification by semi-preparative rplc. As well as partitioning classes of compounds, this approach often resulted in the partial separation of components within given classes of compounds.

Using this method the sesterterpene retronic acid variabilin was isolated from a number of sponges of the order Dic-

TABLE 1. Reverse-Phase Column Fractionation of Sponge Extract

Fraction No.	Eluent	Volume (ml)	Mass (mg)	Activity*
1	H <sub>2</sub> O	100	6746	—
2	H <sub>2</sub> O	100	72	+/-
3	H <sub>2</sub> O-MeOH (3:1)	100	45	—
4	H <sub>2</sub> O-MeOH (3:2)	100	318	+/-
5	H <sub>2</sub> O-MeOH (2:3)	100	191	++
6	H <sub>2</sub> O-MeOH (1:4)	100	83	+
7	H <sub>2</sub> O-MeOH (1:9)	100	63	++
8	MeOH	100	108	++
9	MeOH-CH <sub>2</sub> Cl <sub>2</sub> (9:1)	100	90	+
10	MeOH-CH <sub>2</sub> Cl <sub>2</sub> (3:1)	100	98	—
11	MeOH-CH <sub>2</sub> Cl <sub>2</sub> (1:1)	100	515	—
12	CH <sub>2</sub> Cl <sub>2</sub>	200	604	—
13	MeOH+H <sub>2</sub> O	400	154	—

\*Cytotoxicity to BSC cells in 16-mm wells with a loading of 100 ng/6-mm disk.

++: 2-4 mm zone.

+: 1-2 mm zone.

+/-: minor effects located under disk.

fractions indicated that one compound, a red pigment, was present in all of the cytotoxic fractions. Medium-pressure reverse-phase liquid chromatography (rplc) of these combined fractions, followed by semi-preparative rplc, gave analytically pure discorhabdin C (1), the component responsible for the cytotoxicity of the crude extract. The structure of this novel compound was solved by a single-crystal X-ray diffraction study (5). Fraction 11 was largely sterols, of which the major component was cholesterol (<sup>13</sup>C-nmr spectroscopy) while virtually all the salt was eluted in fraction 1.

When applied to other extracts from sponges, ascidians, and bryozoans, this method cleanly separated the components in the range from salts to sterols, lipids, and carotenoids, leaving them in

ryoceratida (6); the highly oxygenated triterpene thyriferol and the various lauthisan eneynes from the red alga *Laurencia thyrifera* (7,8); 1,3,7-trimethylguanine from the discorhabdins and sterols in New Zealand species of the sponge genus *Latrunculia* (9); oroidin from A-notsterols in a New Zealand species of the sponge genus *Axinella*.<sup>2</sup> Although we have only applied this technique to marine natural products, it is of wider general application.

The possible choice of solvents is wide, while the volumes used are minimal, thus lowering costs and enhancing ease of handling. The time taken for sample preparation and chromatography

<sup>1</sup>Presented, in part, at the PAC CHEM 84 Congress, Honolulu, 16-21 December 1984.<sup>2</sup>Unpublished results, J.W. Blunt and M.H.G. Munro.

is typically about 3 h. The equipment used is inexpensive, and the reverse-phase support may be re-cycled many times, thus offsetting the high initial cost of commercial support. A cheaper alternative, which we have used, is to prepare appropriate reverse-phase packing materials from chromatographic grade silica (3). Finally, this is a high capacity system capable of handling up to about 20 g of crude extract per 100 g of support.

#### EXPERIMENTAL

**MATERIAL.**—Reverse-phase material was prepared by coating Si gel (Woelm, 32–63 microns) with *n*-octadecyltrichlorosilane by the method of Evans *et al.* (3).

**METHOD.**—The MeOH/toluene extract (9.7 g) of a sponge of the genus *Latrunculia*, family Larrunculiidae (voucher no. T430-8) was dissolved/suspended in CH<sub>2</sub>Cl<sub>2</sub>/MeOH/H<sub>2</sub>O (250 ml) and added to the reverse-phase material (5 g). Concentration under reduced pressure gave an aqueous slurry (50 ml) that was added to the top of a column (25 mm i.d.) which had been slurry packed with the same material (50 g) in MeOH and then equilibrated with H<sub>2</sub>O. Details of eluents and fraction volumes are given in Table I. Each fraction was assayed for antiviral/cytotoxic activity (10), but using a BSC cell line rather than CV-1 cells and examining each well microscopically for cytopathic and cytotoxic effects.

#### ACKNOWLEDGMENTS

Financial support from the Harbor Branch Oceanographic Institution (GDF, NBP, VLC and JDM), the New Zealand Lotteries Board (Scientific Distribution Committee) (RJI), the Canterbury Medical Research Foundation, the University of Canterbury, and the New Zealand Universities Grants Committee is gratefully acknowledged.

#### LITERATURE CITED

1. G.R. Pettit, Y. Kamano, R. Aoyagi, C.L. Herald, D.L. Doubek, J.M. Schmidt, and J.J. Rudloe, *Tetrahedron*, **41**, 985 (1985).
2. Y. Shimizu, *J. Nat. Prod.*, **48**, 223 (1985).
3. M.B. Evans, A.D. Dale, and C.J. Little, *Chromatographia*, **13**, 5 (1980).
4. W.C. Still, M. Kahu, and A. Mitra, *J. Org. Chem.*, **43**, 2923 (1978).
5. N.B. Perry, J.W. Blunt, J.D. McCombs, and M.H.G. Munro, *J. Org. Chem.*, **51**, 5476 (1986).
6. N.B. Perry, C.N. Battershill, J.W. Blunt, G.D. Fenwick, M.H.G. Munro, and P.R. Bergquist, *Biochemical Systematics and Ecology*, in press.
7. J.W. Blunt, M.P. Hartshorn, T.J. McLennan, M.H.G. Munro, W.T. Robinson, and S.C. Yorke, *Tetrahedron Letters*, 69 (1978).
8. J.W. Blunt, R.J. Lake, and M.H.G. Munro, *Aust. J. Chem.*, **37**, 1545 (1984).
9. N.B. Perry, J.W. Blunt, and M.H.G. Munro, *J. Nat. Prod.*, **50**, 307 (1987).
10. A.C. Schroeder, R.G. Hughes, Jr., and A. Bloch, *J. Med. Chem.*, **24**, 1078 (1981).

Received 23 July 1986

# EUDISTOMIN K: CRYSTAL STRUCTURE AND ABSOLUTE STEREOCHEMISTRY

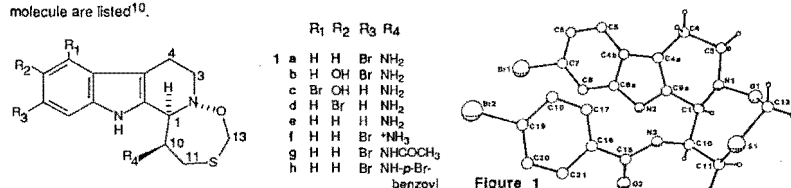
Robin J. Lake, John D. McCombs, John W. Blunt\*, Murray H. G. Munro\* & Ward T. Robinson

Department of Chemistry, University of Canterbury, Christchurch, NEW ZEALAND

**ABSTRACT:** The crystal structure of a derivative of the antiviral and antitumour compound eudistomin K **1a** has been determined, confirming the proposed structure and stereochemistry. The conformation found for the oxathiazepine ring in the solid state is similar to that proposed for the solution state from nmr spectroscopic measurements.

The oxathiazepine eudistomins, first reported from the Caribbean ascidian *Eudistoma olivaceum*<sup>1</sup> **1a-d**, and more recently from the New Zealand ascidian *Ritterella sigillinoides*<sup>2</sup> **1a-b,e**, are tryptophan derivatives of considerable synthetic and biological interest. These eudistomins are distinguished by the unusual structural combination of N, O, C and S, and as a group display potent antiviral and antitumour activity<sup>1,2</sup>. The original structure determination of this new class of alkaloid was based on nmr and mass spectrometric evidence<sup>1</sup>, and supported by a subsequent, extensive nmr spectroscopic study<sup>2</sup>. We now report the crystal structure and confirm the absolute stereochemistry of the *p*-bromobenzoyl derivative of eudistomin K **1h** (Figure 1).

Eudistomin K **1a** was converted to the *p*-bromobenzoyl derivative **1h** by standard methods in good yield<sup>3</sup>. Recrystallisation from methanol/chloroform gave long, thin, tabular, transparent, needles suitable for single crystal X-ray structure analysis. The data were collected using a Nicolet XRD R3 single crystal four-circle diffractometer<sup>4,5</sup>. A Patterson calculation (SHELXS) revealed the position of the four bromine and two sulphur atoms in the asymmetric unit which contains two independent eudistomin K molecules and one methanol molecule. The remaining non-hydrogen atoms were located from difference Fourier syntheses (SHELXTL)<sup>6</sup>. All hydrogen atoms were inserted at calculated positions<sup>7</sup>, except for the hydrogen on the methanol oxygen atom which is potentially able to hydrogen bond to several sites. Since it could not be located in difference Fourier syntheses it was omitted. With the exception of the indolic carbon atoms, all non-hydrogen atoms were assigned anisotropic thermal parameters, and the refinement of 451 least squares parameters converged with  $R = 0.049$  and  $wR = 0.053$ . The final electron density maps showed no significant residual electron density and there were no abnormal discrepancies between observed and calculated structure factors<sup>8</sup>. The absolute configuration was determined unambiguously by eta refinement<sup>9</sup>. Fractional atomic coordinates for both eudistomin K molecules and the methanol solvate molecule are listed<sup>10</sup>.



In the earlier nmr study of the structure and stereochemistry of eudistomin K<sup>2</sup>, **1a**, use was made of the modified Karplus equation<sup>11</sup> to estimate vicinal dihedral angles in the oxathiazepine and  $\beta$ -carboline rings. These data, along with the observation of key nOe derived H/H relationships ( $1\alpha/3\alpha$ ,  $3\beta/13\beta$ ,  $13\alpha/11\alpha$ , but not  $3\alpha/13\alpha$ ,  $3\beta/13\alpha$  or  $11\beta/13\alpha$ ), were critical in the confirmation of the structure of eudistomin K as **1a**, and in proposing the relative stereochemistry and a conformation for eudistomin K **1a** in solution (Figure 2b)<sup>2</sup>. Similar coupling constants and the same nOe relationships were observed for the salt **1f** and the acetyl and *p*-bromobenzoyl derivatives (**1g** and **1h**), indicating the same dominant conformation in solution for the oxathiazepine ring regardless of the polarity and steric bulk of the 10 $\beta$  N function. Although there are two independent molecules of **1h** in the asymmetric unit, they are conformationally equivalent, their slight differences being a

consequence of different packing environments. The conformation of the *p*-bromobenzoyl derivative **1h** in the crystalline state (Figure 2a) is remarkably similar to that proposed for eudistomin K **1a** in solution (Figure 2b) and strongly suggests that the factors determining the conformation of eudistomin K and its derivatives are essentially the same in both the liquid and crystalline state. The most noticeable difference between the conformations is the opening of the N1C1C10N3 dihedral angle as indicated by the estimated dihedral angles for the oxathiazepine ring from the atomic coordinates (**1h**) and coupling constants (**1a**) (Table 1).

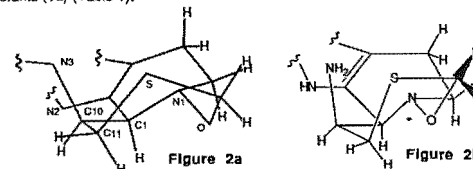


Table 1 Estimated Dihedral Angles

	1a	(J in Hz)	1h
3a/4b	180°	(11.8)	170°
3a/4a	50-60°	(4.0)	50°
3b/4b	50-60°	(4.6)	50°
3b/4a	60-70°	(2.2)	71°
1a/10a	80-90°	(-1)	43°
10a/11a	80-90°	(0)	65°
10a/11b	40°	(6.2)	54°

**ACKNOWLEDGEMENTS:** Support from The Harbor Branch Oceanographic Institution and the NZ UGC is gratefully acknowledged.

## REFERENCES & NOTES

- (a) Rinehart, K. L. Jr., Kobayashi, J., Harbour, G. C., Hughes, R. G. Jr., Mizsak, S. A., and Scabill, T. A., *J. Am. Chem. Soc.*, 1984, 106, 1524-6; (b) Rinehart, K. L. Jr., Kobayashi, J., Harbour, G. C., Gilmore, J., Mascall, M., Holt, T. G., Shield, L. S., and Lafargue, F., *J. Am. Chem. Soc.*, 1987, 109, 3378-87.
- (a) Blunt, J. W., Lake, R. J., Munro, M. H. G., and Toyokuni, T., *Tetrahedron Lett.*, 1987, 28, 1825-26; (b) Lake, R. J., Blunt, J. W., Munro, M. H. G., *Aust. J. Chem.*, (submitted).
- Eudistomin K **1a** (10mg) was reacted with *p*-bromobenzoyl chloride (16mg) in CH<sub>2</sub>Cl<sub>2</sub> (5mL) with added triethylamine (22mg) for 16 hr. The product mixture was purified by normal phase HPLC (silica gel semi-preparative column, 2mL/min CHCl<sub>3</sub>) to give the *p*-bromobenzoyl derivative of eudistomin K **1h** (11mg, 73%), which was recrystallised from methanol/chloroform to give needles, m.p. 192-193°;  $[\alpha]_D^{20} = -200^\circ$  (c 0.1 in CHCl<sub>3</sub>);  $\lambda_{max}$  279nm (ε 10700), 230nm (ε 57500);  $\delta_H$  (CDCl<sub>3</sub> referenced to CHCl<sub>3</sub> at 7.25ppm) 8.72, bs, indole NH; 7.43, 2H, d,  $J = 8.6$ , H18 and H20; 7.37, 2H, d,  $J = 8.6$ , H17 and H21; 7.36, d,  $J = 1.7$ , H8; 7.30, brd,  $J = 9.5$ , benzoyl NH; 7.22, d,  $J = 8.4$ , H5; 7.11, dd,  $J = 8.4$ , 1,7, H6; 5.16, m, H10; 4.99, d,  $J = 9.1$ , H13a; 4.88, d,  $J = 9.1$ , H13b; 4.23, brs, H1; 3.63, ddd,  $J = 10.1$ , 5.1, 1.7, H3b; 3.43, d,  $J = 14.5$ , H11a; 3.17, ddd,  $J = 11.5$ , 10.2, 4.8, H3a; 2.90, dddd,  $J = 15.5$ , 11.5, 5.1, 2.1, H4; 2.88, dd,  $J = 14.5$ , 5.8, H11b; 2.79, m,  $J = 15.5$ , 4.8, 1.7 and small, H4;  $\delta_C$  (CDCl<sub>3</sub> referenced to 77.01ppm) 166.7, C=O; 137.9, C8a; 132.6, C4b; 131.8, C17 and C21; 130.9, C16; 128.5, C18 and C20; 126.5, C9a; 125.0, C19; 122.7, C6; 119.1, C5; 115.5, C7; 114.3, C8; 109.4, C4a; 71.1, C13; 69.0, C10; 54.7, C3; 47.3, C1; 32.3, C11; 20.6, C4.
- Mo K $\alpha$  ( $\lambda$  0.71069 Å) radiation from a crystal monochromator was used. The cell parameters were determined from a least squares refinement of 22 accurately centred high angle reflections ( $19^\circ < 2\theta < 33^\circ$ ). Data were corrected for Lorentz and polarisation effects. An empirical absorption correction, based on  $\psi$ -scan data, was applied. The space group was determined unambiguously as a result of the structure analysis reported below, but initially indicated by systematic absences of the appropriate reflections.
- Crystal data. C<sub>22</sub>H<sub>19</sub>N<sub>3</sub>O<sub>2</sub>SBr<sub>2</sub>, M 535, crystal dimensions 0.8 x 0.4 x 0.1 mm, monoclinic, P2<sub>1</sub>, a 9.994(2), b 2.115(8), c 10.788(2) Å,  $\beta$  104.82(2)°, U 2200(2) Å<sup>3</sup>, D<sub>c</sub> 1.61 cm<sup>-3</sup>, Z 4, F(000) 1072,  $\mu$  (Mo K $\alpha$ ) 3.72 mm<sup>-1</sup>, absorption correction: max 0.755, min 0.414.  $\omega$ -scan intensity measurements ( $4^\circ < 2\theta < 55^\circ$ ), at 133K, yielded 4374 unique reflections 3009 were judged observed ( $I > 3\sigma(I)$ ). Crystal stability was monitored by measuring three check reflections every 500 and no significant variations were observed.
- Sheldrick, G.M., "SHELXTL User Manual", Revision 4.1 (1981) Nicolet XRD Corporation, Madison, Wisconsin, U.S.A., and Sheldrick, G.M., "SHELXS. Program for Crystal Structure Solution", University of Göttingen, Federal Republic of Germany.
- Using a riding model with thermal parameters equal to 1.2U of their carrier atoms.
- Blocked cascade refinements, with reflection weights of  $1/\sigma^2(F) + 0.00084(F^2)$ , and the function minimized was  $\Sigma [F_o - |F_c|]^2$ .
- Rogers, D., *Acta Cryst.*, 1981, A37, 734.  $\eta = 0.95(3)$ .
- Atom Coordinates ( $\times 10^4$ ) and Temperature Factors ( $\text{\AA}^2 \times 10^3$ )

Molecule 1					Molecule 2					Molecule 1					Molecule 2				
Atom	x/a	y/b	z/c	U	Atom	x/a	y/b	z/c	U	Atom	x/a	y/b	z/c	U	Atom	x/a	y/b	z/c	U
BR1	7152(1)	0000(0)	5796(1)	32(1)	1219(1)	5955(1)	10606(2)	26(1)	C7	6418(12)	331(5)	4985(12)	25(3)	10513(12)	5514(5)	10109(12)	23(3)		
BR2	6414(1)	2829(1)	8612(1)	32(1)	12497(1)	3181(1)	14416(1)	32(1)	C8	8802(12)	272(5)	5425(12)	25(3)	8452(12)	5554(5)	10710(12)	25(3)		
S1	1592(3)	2551(2)	5297(3)	26(2)	2893(3)	3429(1)	10350(3)	25(2)	C9a	10671(12)	587(5)	4126(12)	24(3)	6236(12)	5254(5)	10111(12)	23(3)		
N1	1521(2)	1806(4)	3196(8)	25(5)	2831(7)	4187(4)	8476(8)	26(5)	C9b	12462(12)	999(8)	4193(12)	27(3)	6273(12)	4855(5)	9551(13)	25(3)		
O2	13953(8)	1079(4)	7905(8)	28(5)	8207(8)	4617(4)	13490(8)	29(5)	C11	18064(12)	1738(5)	5879(12)	22(7)	3218(11)	4227(5)	11168(12)	22(7)		
N2	13657(10)	1760(4)	3449(9)	22(8)	4145(9)	4100(5)	8714(10)	28(8)	C13	15763(15)	2400(8)	3814(15)	39(8)	2122(13)	3608(8)	8832(13)	35(8)		
N3	12135(9)	578(4)	5074(10)	22(6)	6908(10)	5221(4)	10450(9)	25(8)	C15	13358(12)	1528(5)	7279(12)	23(7)	6550(11)	4285(5)	12773(12)	24(7)		
C3	13765(9)	1740(4)	6250(10)	23(8)	5763(10)	4106(5)	11581(10)	28(8)	C16	12119(11)	1848(5)	7508(12)	24(7)	7958(12)	3827(5)	13126(12)	22(7)		
N1	13951(12)	1183(5)	4277(12)	23(7)	4611(12)	4689(5)	9618(11)	22(7)	C17	11625(12)	2045(5)	8574(12)	26(7)	8579(14)	3837(5)	14427(12)	30(8)		
C3	12893(12)	1672(6)	2103(12)	28(7)	4264(11)	4307(5)	7427(12)	23(7)	C18	8869(12)	2328(5)	8850(12)	27(7)	9947(14)	3608(5)	14848(14)	34(8)		
C4	11396(12)	1874(6)	2347(12)	28(7)	5825(12)	4275(5)	7504(12)	25(7)	C19	9388(12)	2408(5)	8140(12)	24(7)	10820(15)	3469(8)	13912(18)	42(9)		
C4a	11320(11)	1243(5)	3396(11)	20(3)	6703(12)	4650(5)	8839(12)	24(3)	C19	8868(12)	2408(5)	8140(12)	24(7)	10820(15)	3469(8)	13912(18)	42(9)		
C4b	10154(11)	998(5)	3762(12)	21(3)	8048(12)	4901(5)	8972(12)	23(3)	C20	10688(11)	2195(5)	9113(12)	22(7)	13004(12)	3548(5)	12599(13)	28(7)		
C5	8737(12)	1038(5)	3357(13)	26(3)	9172(12)	4650(5)	8420(13)	31(3)	C21	12102(12)	1814(5)	8902(12)	24(7)	8650(12)	3786(8)	12224(13)	32(8)		
C8	7884(13)	795(5)	3933(13)	29(3)	10398(13)	5174(5)	8971(13)	28(3)											
Cm	5638(15)	2481(8)	711(15)	45(4)					Om	8042(8)	3124(4)	234(8)	32(2)						

Structure factor and molecular dimension tables have been deposited with the Cambridge Crystallographic Data Centre.

- Haasnoot, C. A. G., de Leuw, F. A. A. M., and Altona, C., *Tetrahedron*, 1980, 36 2783-92.

(Received in UK 26 July 1988)

## Novel 2(5H)-Furanones from the Red Marine Alga *Delisea elegans* (Lamouroux).

John D. McCombs, John W. Blunt\*, Mark V. Chambers, Murray H.G. Munro\*, Ward T. Robinson.

Department of Chemistry, University of Canterbury, Christchurch, New Zealand.

(Received in UK 19 October 1987)

**ABSTRACT** - Six novel 2(5H)-furanones, all related to the previously reported 'limbrolide' 3-butyl-4-bromo-5-(dibromomethylidene)-2(5H)-furanone, have been isolated from the red marine alga *Delisea elegans* (family Bonnemaisoniaceae). Three of the six compounds, characterised by spectroscopic and single crystal X-ray structure analyses, contain unusual poly-brominated cyclobutane functions.

### INTRODUCTION

Red marine algae from the family Bonnemaisoniaceae have been shown to produce a wide range of halogenated metabolites,<sup>1</sup> including butenones, acetones, acrylic and acetic acids,<sup>2,3,4</sup> pyranones, octenones,<sup>5</sup> and from the genus *Delisea*, halogenated 2(5H)-furanones.

A series of 2(5H)-furanones, named limbrolides, with the structures (1) through (4)<sup>6</sup> were isolated from the dichloromethane soluble material of extracts of *D. fibriata*, collected on the east coast of Australia. A similar study of *D. fibriata* collected from Antarctica described the isolation of 2(5H)-furanones (1a) - (1d), (1f) and (1g),<sup>7</sup> together with a series of oct-1-en-3-ones (5a) - (5e).<sup>8</sup>

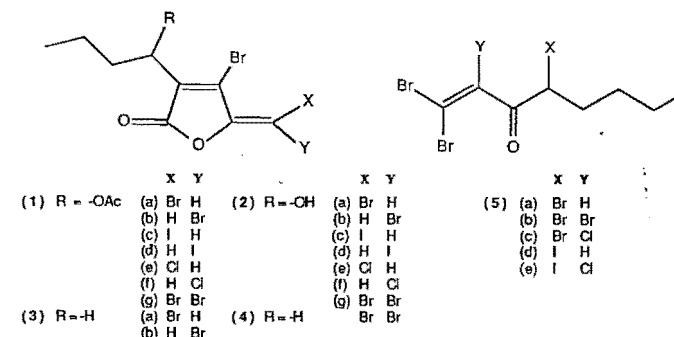
In both cases interest in the species was due to observations of biological activity. In the first instance high *in vitro* antimicrobial activity was found,<sup>6</sup> while in the second, plants were observed to be remarkably free of epibionts.<sup>7,8</sup>

In this present study six new metabolites were isolated from *Delisea elegans* collected at Kaikoura, New Zealand. The previously reported furanone (4) was identified as the major component of the mixtures, and six novel halogenated furanones, (6), (7), (9) - (12), all related to (4), were found.

### DISCUSSION

Extraction of air dried *Delisea elegans*, collected from Kaikoura in April 1981, afforded a 5% organic extract (extract A). Initial open-column chromatography on silica gel, and subsequent HPLC resulted in the isolation of compounds (4), (7), (10), (11) and (12). Using similar procedures the metabolites (6) and (9) were obtained from an extract (extract B) of the alga collected from the same site in April 1983.

Comparison of the crude organic soluble extracts A and B by HPLC showed that they both contained all of the metabolites identified, although in slightly differing amounts.

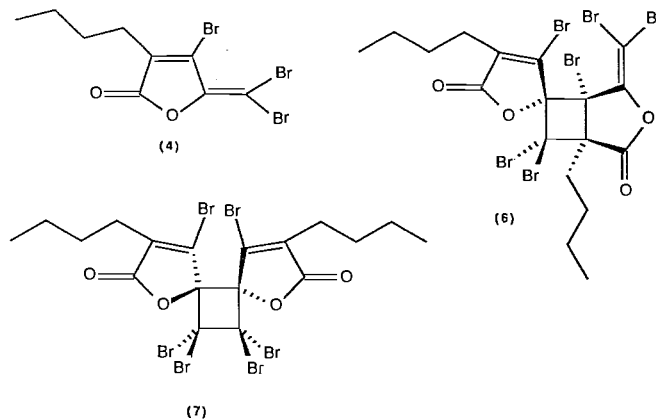


The least polar compound in the extracts was heptadecane, which was identified by GC and <sup>13</sup>C-NMR comparisons with authentic samples.

The major component of the extracts, also among the least polar of the compounds, was the previously reported furanone (4). This compound was estimated to represent 80% of the organic soluble material in each of the extracts.

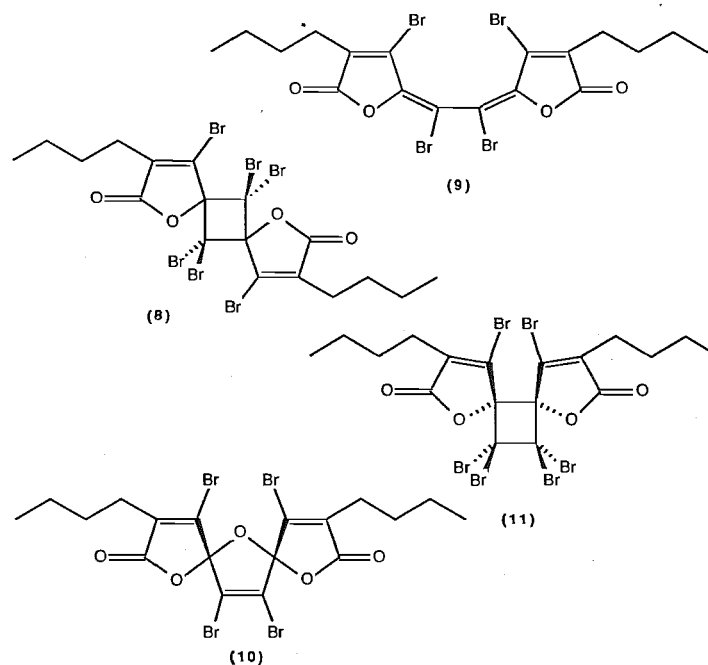
A more polar compound ( $\pm$ )-*trans*-4,10,11,11,12,12-hexabromo-3,9-dibutyl-1,7-dioxaspiro[4.0.4.2]dodeca-3,9-diene-2,8-dione (7) was obtained from a silica gel column fraction of extract A by HPLC. The spectroscopic data indicated a strong structural similarity to (4). The <sup>13</sup>C-NMR spectrum of (7) contained nine resonances, an n-butyl group ( $\delta_c$  28.43, 25.69, 22.06, 13.59), a carbonyl group ( $\delta_c$  165.56), two olefinic carbon atoms ( $\delta_c$  140.09, 136.66), and two further quaternary resonances ( $\delta_c$  89.79, 69.88). The only resonances in the <sup>1</sup>H-NMR spectrum were associated with the n-butyl group. The strong carbonyl absorption (1800 cm<sup>-1</sup>) was characteristic of the  $\alpha,\beta$ -unsaturated furanone system in compounds (1) - (4), while the UV absorption maximum (249nm) suggested a singly conjugated enone system. Finally the observation of a single quintet (Br<sub>4</sub>) observed in the low resolution FAB mass spectrum at *m/z* 615 (C<sub>18</sub>H<sub>19</sub>Br<sub>4</sub>O<sub>4</sub>) suggested a dimeric structure for compound (7), with a requirement for a high degree of symmetry. The compound was eventually crystallized as colourless needles, and the structure solved using single crystal X-ray methods.

A more polar column fraction from extract A contained two major components, in addition to some minor contaminants. The minor components were removed by HPLC, and fractional recrystallisation from pentane yielded crystals of two distinct types, one with needle-like crystals, and the other with plate-like ones.



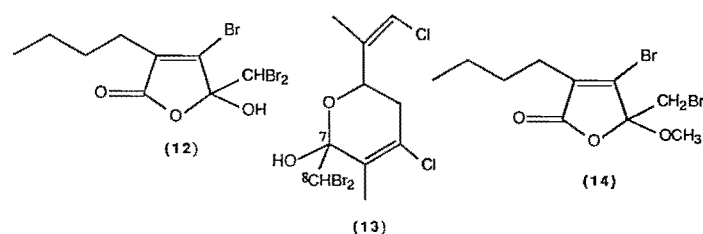
The compound with needle-like crystals was shown to be *cis*-4,11,12,13-tetrabromo-3,10-dibutyl-1,6,8-trioxadispiro[4.1.4.2]trideca-3,10,12-triene-2,9-dione (10) by single crystal X-ray structure analysis. High thermal motion, or disorder, in the terminal n-butyl groups was indicated by the very large temperature factors for these atoms. Many attempts were made, using disordered models, to account more satisfactorily for the geometry of the C7', C8' and C9' moiety. None were better than that implicit in table 1, and the extreme difficulties involved in obtaining more material precluded recollection of the X-ray data at low temperature. No useful spectroscopic data could be obtained from the small amount of material available.

The compound which crystallised as plate-like crystals was identified as ( $\pm$ )-*cis*-4,10,11,11,12,12-hexabromo-3,9-dibutyl-1,7-dioxadispiro[4.0.4.2]dodeca-3,9-diene-2,8-dione (11). The infrared spectrum of the compound contained a strong carbonyl band ( $1800\text{cm}^{-1}$ ) characteristic of the 2(5H)-furanone function, while the  $^1\text{H-NMR}$  spectrum on comparison with (4) indicated the presence of an n-butyl chain. The carbonyl ( $\delta_c$  165.57) and the olefinic resonances ( $\delta_c$  142.57, 136.37) were very similar to those of the cyclobutane dimer (7), indicating a closely related structure. Comparison of the two remaining singlet resonances ( $\delta_c$  86.42, 59.08) with those of the dimer (7) suggested that the structure was either the *cis* diastereomer (11) of (7), or the [4.1.4.1] (8) structural isomer. A room temperature single crystal X-ray structure analysis showed unambiguously that the structure was the diastereomer (11). The only small single crystal sample was of very poor quality, and could not be improved with the limited material available. The present refinement level of  $R=0.11$ , (owing to disorder in the n-butyl chains) is not yet good enough to warrant publication of further detail in this paper.



Preparative HPLC of the most polar silica gel column fractions, from extract A, yielded the 3-butyl-4-bromo-5-hydroxy-5-(dibromomethyl)-2(5H)-furanone (12). High resolution mass spectrometry indicated a molecular formula of  $\text{C}_9\text{H}_{11}\text{Br}_3\text{O}_3$  ( $m/z$  404,  $\text{Br}_3$ ) which allowed for three double bond equivalents. The observed loss of 18 AMU is characteristic of an alcohol function, while the resulting ion cluster at  $m/z$  387 ( $\text{Br}_3$ ) suggested that the compound was closely related to (4). The presence of an alcohol function was confirmed by the infrared spectrum which showed a sharp absorption band at  $3525\text{cm}^{-1}$ . A strong carbonyl band at  $1790\text{cm}^{-1}$  was further evidence for the similarity of structure to (4). The UV absorption maximum (235nm) was consistent with a 3,4-unsaturated 2-furanone. Comparison of the  $^1\text{H-NMR}$  spectrum with that of (4) showed that nine of the eleven protons were associated with an n-butyl chain. The hydroxyl proton was observed as a broad singlet at  $\delta_H$  4.65. The remaining proton, a sharp singlet at  $\delta_H$  5.85, was assigned by comparison with costatone (13)<sup>9</sup>,  $\delta_H$  5.80, as belonging to a dibromomethyl group. The  $^{13}\text{C-NMR}$  multiplicities were consistent with the eleven proton integral. Comparison of the chemical shifts of the n-butyl and olefinic resonances with those of (4) ruled out the possibility of the 3- or 4 position being substituted by either the hydroxyl or dibromomethyl groups. This together with the other spectroscopic data indicated the presence of a 3-butyl-4-bromo-2(5H)-furanone moiety. This assignment of the substitution of the olefin implied that both the hydroxyl and dibromomethyl groups

were placed  $\gamma$  to the carbonyl group, i.e. structure (12). ORD measurements showed that the compound was a racemic mixture.



Further proof of the identity of this compound was obtained by reaction of (4) with aqueous potassium hydroxide in tetrahydrofuran. Analysis by HPLC of the reaction mixture indicated a product with an identical retention volume to (12). This reaction is analogous to that reported by Pettus *et al*<sup>7</sup> in which 3-butyl-4-bromo-5-(bromomethylidene)-2(5H)-furanone (3a) or (3b) was reacted with methanolic potassium hydroxide to give the corresponding methoxyl derivative (14).

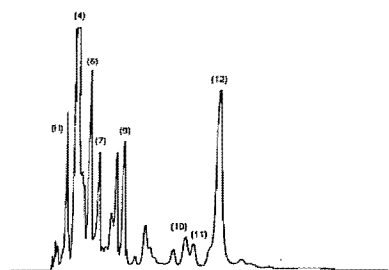


Figure 1. HPLC chromatogram of extract B (cyanopropyl, 1% propan-2-ol/hexane, 210nm). The peaks are marked with compound numbers (H - heptadecane).

The two new compounds isolated from extract B of *D. elegans*, collected in April 1983, were characterised by single crystal X-ray structure determinations as 3,5',7',7'-tetrabromo-1',4-dibutyl-4'-(dibromomethylene) spiro[furan-2(5H),6'-[3]oxabicyclo[3.2.0]heptane]-2',5-dione (6) and (Z,Z)-5,5'-(1,2-dibromo-1,2-ethanediylidene)bis[4-bromo-3-butyl-2(5H)-furanone] (9).

A number of structural and stereoisomers of the compounds described here are possible. Several other compounds were isolated whose <sup>13</sup>C-NMR spectra indicated they were closely related to the compounds described here, but lack of crystals and poor spectroscopic data prevented their structural elucidation. The compounds isolated are shown in an HPLC chromatogram of extract B (figure 1).

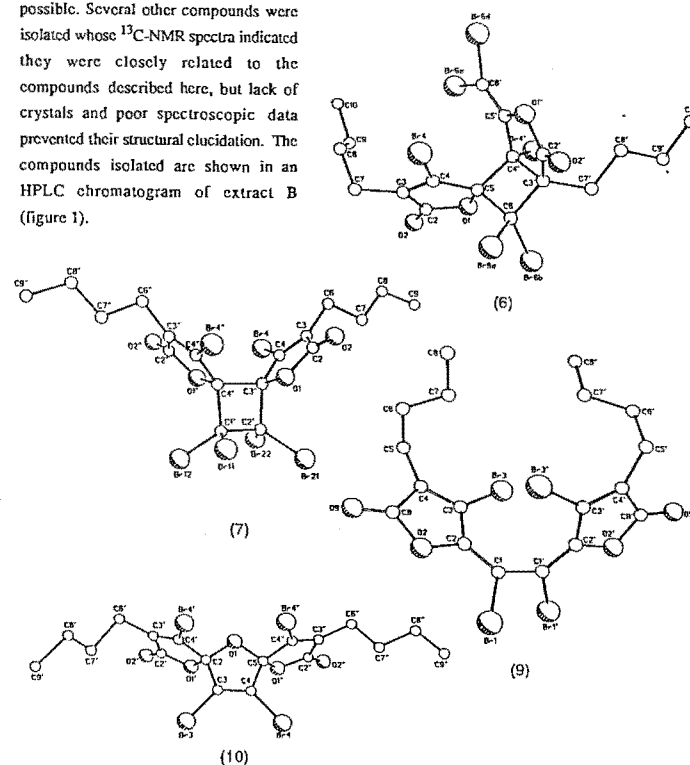


Figure 2. Perspective Drawings of compounds (6), (7), (9) and (10). Note (7) lies on a crystallographic two-fold axis.

From a spectroscopic view point these compounds presented considerable problems. In spite of using FAB and FD ionisation techniques reliable mass spectra proved almost impossible to obtain. The only molecular ions observed were for the compounds (10) and (12). The highly substituted nature of these structures meant the <sup>1</sup>H-NMR spectra contained little useful information, while the long relaxation times for many of the carbon atoms made it difficult to record adequate <sup>13</sup>C-NMR spectra with the limited quantities of compound available.

Computer generated perspective drawings of (6), (7), (9) and (10) are shown in figure 2. Only non-hydrogen atoms are shown. Table 1 shows the fractional atomic coordinates for these compounds. A crystallographically convenient system of atom numbering has been used for these diagrams and tables and in the deposited material.

#### Comparison of collections.

A total of seven collections of *D. elegans* were made, at times which spanned most of the observed growing season at Kaikoura (mid October to late April). To check whether there was a geographical or seasonal variation in the types of compounds found, extracts of samples from each collection were made using a standard technique, and compared by HPLC.

Dried, powdered samples of the alga were extracted with dichloromethane, and filtered through short "Florisil" columns in ether to remove pigments and other polar materials. The resulting oils were analysed by HPLC on a cyanopropyl column. Examination of the chromatograms revealed variations of the relative concentrations of compounds, but no significant qualitative differences were found.

#### Origin of the compounds.

To ensure that the compounds described here were not artifacts, especially of photochemical origin, a collection of *D. elegans* was made in March 1982. The alga was placed in an opaque plastic bag before being brought to the surface. The subsequent extraction and analysis procedures were performed in total darkness to prevent any possible photo-dimerisation. To eliminate the possibility of artifacts being caused by the extraction and column chromatographic methods the alga was simply homogenised with dichloromethane, and the centrifuged extract immediately analysed by HPLC. Comparison of the chromatogram obtained by this method with those of extracts from other collections revealed no significant qualitative difference, supporting the view that the compounds described here are of natural origin.

The biochemical origin of the compounds described is unclear. The cyclobutane containing dimers (6), (7) and (11) could all be derived from (4) by  $[\pi_2s + \pi_2s]$  cycloaddition reactions. Compound (10) can be derived from a condensation between (4) and (12), while the dimer (9) could have arisen from the appropriate vinyl radicals. Compound (12) may be formed by the addition of water across the exocyclic double bond of (4) or alternatively (12) could be the precursor of (4).

Molecules which are formed by enzyme mediated reactions are almost invariably optically active.<sup>10</sup> Examples of cyclobutane-containing dimers are known. Optically active scepterin (15) is related to the achiral debromooroidin (oroidin (16) by a  $[\pi_2s + \pi_2s]$  cycloaddition reaction. Although this cycloaddition reaction is photochemically allowed attempts to dimerise oroidin failed and a biosynthetic origin was postulated.<sup>11</sup> In contrast all of the compounds isolated here were either *meso* or racemic mixtures. This lack of stereospecificity in the biosynthesis of these compounds suggests they may be formed photochemically *in situ*.

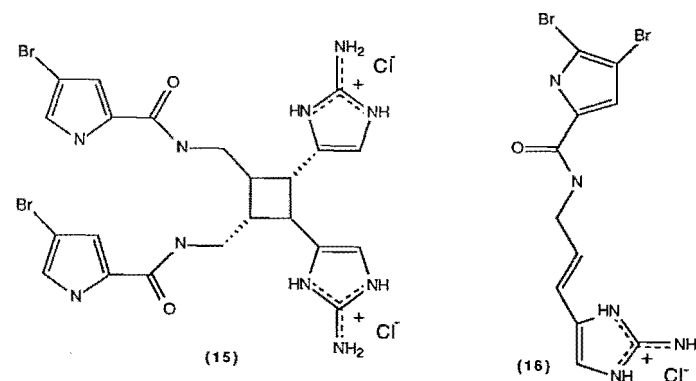


Table 1. Fractional coordinates for atoms in compounds (6), (7), (9) and (10). The equivalent isotropic temperature factor in this table is defined as one third of the trace of the orthogonalised U tensor.

Atom	10 <sup>4</sup> X/a	10 <sup>4</sup> Y/b	10 <sup>4</sup> Z/c	10 <sup>3</sup> U	Atom	10 <sup>4</sup> X/a	10 <sup>4</sup> Y/b	10 <sup>4</sup> Z/c	10 <sup>3</sup> U
<i>3,5',7',7'',-Tetrabromo-1',4'-dibutyl-4''-(dibromomethylene)spiro[furan-2(5H),6'-[3]oxabicyclo[3.2.0]heptane]-2',5'-dione (6).</i>									
Br(4)	-943(2)	6700(2)	7954(1)	47(1)	C(6)	2421(15)	7088(14)	6860(6)	37(4)
Br(4')	4684(2)	3655(2)	7293(1)	48(1)	C(7)	1940(21)	8773(17)	9373(7)	58(6)
Br(6a)	845(2)	8662(2)	6681(1)	63(1)	C(8)	1688(17)	7553(19)	9855(7)	57(6)
Br(6b)	4820(2)	8120(2)	6643(1)	62(1)	C(9)	3401(26)	6937(19)	10177(8)	83(8)
Br(6c)	2210(2)	2819(2)	8604(1)	66(1)	C(10)	2870(20)	5563(19)	10625(8)	70(6)
Br(6d)	-1724(2)	1367(2)	7756(1)	58(1)	C(2)	-123(15)	4612(14)	6317(6)	36(4)
O(1)	4610(11)	7172(10)	7982(4)	45(3)	C(3)	1963(14)	5255(14)	6492(6)	31(4)
O(1')	-546(11)	3497(10)	6740(4)	46(3)	C(4)	2555(14)	4717(13)	7201(6)	29(4)
O(2)	5893(12)	8705(12)	9034(5)	60(4)	C(5)	871(13)	3543(13)	7290(6)	34(4)
O(2')	-1179(11)	4895(11)	5860(4)	56(4)	C(6)	556(19)	2716(14)	7797(6)	47(5)
C(2)	4525(22)	8071(15)	8644(7)	51(6)	C(7)	2764(20)	4593(14)	5856(6)	49(5)
C(3)	2628(17)	8003(14)	8714(6)	39(5)	C(8)	2116(17)	2726(15)	5540(6)	46(5)
C(4)	1573(18)	7149(14)	8120(6)	41(5)	C(9)	3249(20)	2089(15)	4997(7)	55(6)
C(5)	2791(13)	6598(14)	7599(6)	30(4)	C(10)	2644(19)	222(16)	4697(7)	56(6)
<i>(±)-trans-4,10,11,11,12,12-Hexabromo-3,9-dibutyl-1,7-dioxaspiro[4.0.4.2]dodeca-3,9-diene-2,8-dione (7).</i>									
Br(21)	1184(2)	-608(2)	7126(2)	73(2)	C(4)	125(12)	2362(15)	6272(14)	42(12)
Br(22)	-876(2)	-172(2)	5678(2)	66(2)	C(6)	1027(15)	3853(17)	5826(17)	66(16)
Br(4)	-1024(1)	2603(2)	5109(2)	61(2)	C(7)	1319(20)	3400(21)	4978(20)	95(21)
O(1)	1240(1)	1720(10)	7860(9)	50(9)	C(8)	1400(22)	4294(23)	4253(22)	105(28)
O(2)	2370(10)	2810(10)	7930(10)	72(12)	C(9)	1675(34)	3898(32)	3359(28)	181(53)
C(2)	1605(20)	2530(18)	7435(20)	57(15)	C(2)	116(13)	225(15)	7006(15)	51(13)
C(3)	876(10)	2901(20)	6450(15)	51(14)	C(3)	279(12)	1544(14)	7161(13)	44(13)
<i>(Z,Z)-5,5'-(1,2-Dibromo-1,2-ethanediylidene)bis[4-bromo-3-butyl-2(5H)-furanone] (9)</i>									
Br(1)	9108(2)	824(1)	7332(1)	53(1)	C(6)	1339(15)	2013(11)	9729(7)	61(4)
Br(3)	3280(1)	2173(1)	6700(1)	50(1)	C(7)	2257(18)	3270(11)	9394(8)	73(5)
Br(1')	7492(2)	295(1)	5026(1)	57(1)	C(8)	2353(22)	4123(14)	10195(10)	102(7)
Br(3')	7404(2)	3035(1)	8073(1)	54(1)	C(9)	3962(14)	-482(10)	9076(6)	46(4)
O(2)	5606(9)	-667(6)	8523(4)	48(2)	C(1')	7428(12)	1189(9)	6131(6)	38(3)



Table 1. continued.

Atom	10 <sup>4</sup> X/a	10 <sup>4</sup> Y/b	10 <sup>4</sup> Z/c	10 <sup>3</sup> U	Atom	10 <sup>4</sup> X/a	10 <sup>4</sup> Y/b	10 <sup>4</sup> Z/c	10 <sup>3</sup> U
O(9)	3606(10)	-1149(8)	9838(5)	67(3)	C(2)	7601(13)	2492(10)	6055(6)	42(3)
O(2)	7796(9)	3216(6)	5128(4)	53(3)	C(3)	7567(12)	3478(9)	6729(6)	40(3)
O(9)	8007(12)	5389(8)	4529(6)	81(4)	C(4)	7651(14)	4710(10)	6264(7)	52(4)
C(1)	7139(13)	300(8)	7046(6)	37(3)	C(5)	7601(16)	6081(10)	6592(9)	70(5)
C(2)	5663(13)	218(9)	7674(6)	40(3)	C(6)	5778(20)	6745(11)	6635(10)	84(6)
C(3)	3980(13)	928(9)	7690(6)	40(3)	C(7)	4445(22)	6115(14)	7404(10)	96(7)
C(4)	2976(13)	589(10)	8536(6)	44(3)	C(8)	2656(18)	6838(14)	7463(10)	90(6)
C(5)	1223(13)	1102(11)	8953(7)	51(4)	C(9)	7819(15)	4567(10)	5232(8)	57(4)

*cis*-4,11,12,13-Tetrabromo-3,10-dibutyl-1,6,8-trioxadispiro[4.1,4.2]trideca-3,10,12-triene-2,9-dione (10).

Br(3)	6112(1)	8579(1)	697(2)	75(1)	C(3)	6604(8)	10927(9)	1314(22)	91(15)
Br(4)	7838(1)	7466(1)	-998(2)	90(1)	C(4)	7099(7)	10334(8)	1544(16)	67(10)
Br(4')	7581(1)	9996(1)	3514(2)	84(1)	C(6)	6250(10)	11528(12)	2497(24)	132(18)
Br(4'')	9395(1)	8941(1)	1738(2)	67(1)	C(7)	5357(14)	1043(15)	3175(33)	178(29)
O(1)	8064(5)	9944(5)	-493(11)	68(6)	C(8)	5134(20)	11637(15)	3635(46)	404(87)
O(1')	6758(6)	10285(6)	-1187(12)	90(8)	C(9)	4205(12)	11194(13)	4383(27)	152(20)
O(2)	5922(7)	11358(7)	-1175(16)	136(2)	C(2')	9210(9)	9062(9)	-3248(17)	76(11)
O(1'')	8437(5)	9215(5)	-2840(9)	74(7)	C(3')	9703(8)	8934(7)	-1733(14)	60(9)
O(2')	9428(6)	9031(7)	-4633(13)	103(10)	C(4')	9198(8)	9023(7)	-518(15)	66(10)
C(2)	7263(7)	9869(7)	-20(16)	65(9)	C(6')	10563(7)	8749(8)	-1736(16)	72(10)
C(3)	7090(7)	8976(9)	-28(16)	67(10)	C(7')	10725(7)	7834(8)	-1814(15)	72(10)
C(4)	7697(8)	8576(8)	-639(15)	66(8)	C(8')	11618(9)	7636(9)	-1761(18)	89(11)
C(5)	8336(7)	9182(8)	-1075(13)	63(9)	C(9')	11780(10)	6742(10)	-1771(19)	111(16)
C(2')	6373(9)	10922(11)	-452(21)	90(14)					

## EXPERIMENTAL

**General Experimental.** Preparative and analytical high pressure liquid chromatography (HPLC) was conducted using a Varian 5020 Liquid Chromatograph, a Varian UV-50 UV/Visible detector, and an Hewlett Packard 3390A Integrating Recorder. All chromatograms were obtained using Du Pont Zorbax bonded phase micro-particle columns. The solvents used were either Waters chromatographic quality, or carefully purified<sup>12</sup> and dried prior to use. Pet. ether refers to light petroleum (boiling range 50–70°C), and ether to diethyl ether. A Varian 3770 Gas Chromatograph with an FID and an Hewlett Packard 3390A integrating recorder were used for the gas chromatography. Redistilled and dried solvents were used for column chromatography. Thin layer chromatograms were obtained using deactivated silica gel G (Stahl), 0.5mm thick on glass plates. The plates were visualised by either spraying with iodine solution (1% w/v, methanol), or by baking at 100° after spraying with phosphomolybdic acid solution (10% w/v, ethanol), or 2,4-dinitrophenylhydrazine solution (0.1g 2,4-DNP, 100ml ethanol, 1ml HCl). Fast atom bombardment mass spectroscopy (FAB) was performed on an MS-80 mass spectrometer using xenon as the ionisation gas, and glycerol as the dispersion matrix. Field desorption spectra (FD) were obtained using a Varian MAT 720 mass spectrometer. The high resolution EI spectrum of (12) was obtained with a VG 7070F spectrometer. Carbon-13 nuclear magnetic resonance (<sup>13</sup>C-NMR) spectra, and proton nuclear magnetic resonance (<sup>1</sup>H-NMR) spectra were obtained with a Varian CFT-20 instrument. The multiplicities of <sup>13</sup>C-NMR spectra were established using either single frequency offset resonance decoupling (SFORD) or 'attached proton test' (APT) techniques.<sup>13</sup> Infrared spectra were recorded using

either a Pyc Unicam SP3-300 Infrared Spectrophotometer, or a Shimadzu IR27G spectrophotometer. Samples were run as carbon tetrachloride solutions in a 10mm sodium chloride cell. Ultraviolet spectra were recorded on a Varian Super Scan 3 Ultraviolet-visible spectrophotometer. Melting points were obtained using a Reichert 'Kofler' hot stage microscope and are uncorrected. Optical rotatory dispersion (ORD) spectra were recorded on a Jasco Model ORD/UV-5 spectrophotometer.

**Isolation of Metabolites from Extract A.** *Delisea elegans* was collected sublittorally (-15m) from St Kilda Rocks, Kaikoura, 160km north of Christchurch on the 2nd of April 1981. The alga was frozen and subsequently air dried, ground and extracted with dichloromethane in a Soxhlet apparatus. The extraction yielded a dark green-brown odiferous oil (3.8g; 4.6% of 82g dry weight).

An analysis by HPLC (cyanopropyl 4.5 x 250mm; heptane/propan-2-ol (5%), 210nm) resolved more than eleven components.

The organic soluble oil (2.5g) was chromatographed on "Florisil" (Sigma 100-200 mesh) (120g; 25 x 500mm column). Fifty fractions of 25ml were collected with increasing solvent polarity (fractions 1 to 4, pentane; 5 to 6, pet. ether; 7 to 10, 0.25% dichloromethane/pet. ether; 11 to 15, 1.0%; 16 to 17, 2%; 18 to 20, 5%; 21 to 25, 10.0%; 26 to 31, 20%; 32 to 39, 50%; 40 to 45, 100%; 46 to 50, ether).

Each fraction was analysed by TLC and fractions with similar composition were combined as follows: 1 to 4 (21mg), 5 to 6 (15mg), 7 to 10 (23mg), 11 to 17 (600mg), 18 to 29 (358mg), 30 to 35 (52mg), 36 to 37 (13mg), 38 to 42 (95mg), 43 to 45 (41mg), 46 to 47 (468mg), 48 to 50 (24mg).

**Isolation of 3-butyl-4-bromo-5-(dibromomethylidene)-2-(5H)-furanone (4).** Fractions "11" to "17" (585mg) all showed one major component by TLC ( $R_f$  = 0.65; pet. ether/ether 10%). This component (200mg) was subjected to preparative HPLC (cyanopropyl 9.5 x 250mm; heptane/propan-2-ol (1%); 220nm), which yielded a very pale yellow, sweet smelling oil (160mg; > 98% pure) which was identified as (4).<sup>14</sup> (4): IR (CHCl<sub>3</sub>) 1790 cm<sup>-1</sup>; EIMS  $m/z$  385 (C<sub>9</sub>H<sub>9</sub>Br<sub>3</sub>O<sub>2</sub>); <sup>1</sup>H-NMR (CDCl<sub>3</sub>)  $\delta_H$  2.4 (t, J=7Hz, 2H), 1.1 to 1.7 (m, 4H), 0.92 (t, J=7Hz, 3H); <sup>13</sup>C-NMR  $\delta_C$  164.78 (s), 144.71 (s), 138.07 (s), 128.35 (s), 81.44 (s), 28.80 (t), 25.82 (t), 22.46 (t), 13.71 (q); UV  $\lambda_{max}$  cyclohexane (e) 308nm (16000).

**Isolation of (±)-trans-4,10,11,11,12,12-Hexabromo-3,9-dibutyl-1,7-dioxadispiro[4.0,4.2]dodeca-3,9-diene-2,8-dione (7).** High pressure liquid chromatography (cyanopropyl 4.5 x 250mm; heptane/propan-2-ol (2%); 220nm) of the combined fractions "18" to "29" (358mg) revealed five major components. These five components were separated from the mixture (190mg) using preparative HPLC (cyanopropyl 9.5 x 250mm; heptane/propan-2-ol (1%); 270nm). Colourless crystals (44mg) were obtained from the second fraction eluted. (7): m.p. 102–104°C; IR 1800cm<sup>-1</sup>; <sup>1</sup>H-NMR (CDCl<sub>3</sub>)  $\delta_H$  2.42 (t, J=7Hz, 4H), 1.1 to 1.7 (m, 8H), 0.93 (t, J=7Hz, 6H); <sup>13</sup>C-NMR (CDCl<sub>3</sub>)  $\delta_C$  165.56 (s), 140.09 (s), 136.66 (s), 89.79 (s), 69.88 (s), 28.43 (t), 25.69 (t), 22.06 (t), 13.59 (q); UV  $\lambda_{max}$  cyclohexane (e) 249nm (15400).

**Preparative High Liquid Chromatography of Combined Fractions "38" to "42".** This fraction contained two major components of similar HPLC retention volumes, contaminated with several minor components. Initial purification was by HPLC (cyanopropyl 4.5 x 250mm; hexane/propan-2-ol (5%),

220nm), and fractional recrystallisation from pentane yielded two compounds, one with needle-shaped crystals (10), and the other with plate-like crystals (11).

*cis-4,11,12,13-Tetrabromo-3,10-dibutyl-1,6,8-trioxadisp[4.1.4.2]trideca-3,10,12-triene-2,9-dione* (10). The compound with needle-like crystals was identified by X-ray crystallography as (10). FABMS  $m/z$  630 ( $C_{18}H_{18}O_5Br_4$ ).

*(±)-cis-4,10,11,11,12,12-Hexabromo-3,9-dibutyl-1,7-dioxadisp[4.0.4.2]dodeca-3,9-diene-2,8-dione* (11). The compound with plate-like crystals was identified as the furanone dimer (11): m.p. 153–154°C; IR 1800  $cm^{-1}$ ;  $^1H$ -NMR ( $CDCl_3$ )  $\delta_H$  2.0 (t,  $J=7Hz, 4H$ ), 1.1 to 1.8 (m, 8H), 0.95 (t,  $J=7Hz, 6H$ );  $^{13}C$ -NMR  $\delta_C$  165.57 (s), 142.57 (s), 136.37 (s), 86.42 (s), 59.08 (s), 28.65 (t), 26.41 (t), 22.35 (t), 13.7 (q); UV  $\lambda_{max}$  cyclohexane ( $\epsilon$ ) 246nm (8750).

*3-Butyl-4-bromo-5-dibromomethyl-5-hydroxy-2-(5H)-furanone* (12). Column fractions "46" to "50" were shown to contain one major component by TLC (silica; pet. ether/ether 30%) and analytical HPLC (cyanopropyl 4.5mm x 250mm; heptane/propan-2-ol (20%); 220nm). Preparative HPLC (cyanopropyl 9.5 x 250mm; heptane/propan-2-ol; 220nm) of the combined fractions (180mg) gave the hydroxy-furanone (12) as a viscous pale brown-yellow oil (102mg). (12): IR 3525, 1790  $cm^{-1}$ ; the ORD spectrum indicated no optical activity;  $^1H$ -NMR ( $CDCl_3$ )  $\delta_H$  5.85 (s, 1H), 4.65 (bs, 1H), 2.38 (t,  $J=7Hz, 2H$ ), 1.1 to 1.7 (m, 4H), 0.93 (t,  $J=7Hz, 3H$ );  $^{13}C$ -NMR  $\delta_C$  168.03(s), 139.27 (s), 137.41 (s), 102.59 (s), 44.44 (d), 28.57 (t), 24.81 (t), 22.32 (t), 13.63 (q); HREIMS  $m/z$  405.82202 ( $C_9H_{11}O_3^{79}Br_2^{81}Br$ ) requires 405.8257; UV  $\lambda_{max}$  cyclohexane ( $\epsilon$ ) 235nm (10000).

To 1.5mg of (4), dissolved in tetrahydrofuran, three drops of concentrated aqueous potassium hydroxide were added. The reaction mixture was neutralised with HCl, and extracted with dichloromethane (3x3ml). The concentrated extract was compared with the original crude extract by HPLC (cyanopropyl column 4.5 x 250mm; hexane/propan-2-ol 5%; 220nm). The reaction showed one major component whose retention volume corresponded to the hydroxy-furanone (12), and several other minor components.

*Isolation of Compounds from Extract B.* A second substantial collection of *Delisea elegans* was made from St Kilda Rocks, in April 1983, and stored by freezing. The frozen alga (4kg) was air dried in darkness (300g dry weight), ground and extracted with ether, for 24 hours, in a Soxhlet apparatus. The green brown odiferous oil (10g) was chromatographed on a silica gel filtration column (50mm deep, 150mm dia., 350g Grace 923 silica gel), eluting with solvent mixtures ranging from pet. ether through to ether: pet. ether 1.5: 0.5%, 5%, 10%, 20%, 50%, 100% ether, 1.0l each.

The three initial fractions (39mg), were shown to be heptadecane. Identification was by  $^{13}C$ -NMR.  $\delta_C$  32.04, 29.78, 22.70, 14.02;<sup>15</sup> HREIMS  $m/z$  240.2819 ( $C_{17}H_{35}$ ) requires 240.2817. The identification was confirmed by capillary GC comparison with standard n-alkanes, using a semilog plot of retention time against chain length; column, DB-1 (J&W);  $C_{16}$ , 3.69 min;  $C_{18}$  6.13 min;  $C_{19}$  8.26 min;  $C_{20}$  11.40 min;  $C_{22}$  22.77 min; unknown 4.67 min. A calculated retention time for  $C_{17}H_{35}$  was 4.69 min.

The fourth and fifth fractions (3.4g) were mixtures of 3-butyl-4-bromo-5-(dibromomethylidene)-2(5H)-furanone (4) and more polar compounds. The two fractions were chromatographed by HPLC

(cyanopropyl; 0.1% propan-2-ol/hexane) to yield pure (4) (440mg) and a mixture of more polar compounds (85mg). Further HPLC of the mixture of polar compounds, under similar conditions, afforded two pure crystalline compounds (30mg, 15mg). Crystallisation was achieved by dissolving the material in pet. ether/ether and allowing the solvents to evaporate slowly at 4°C. The structures of both compounds were determined by X-ray crystallography.

*3,5',7',7'',-Tetrabromo-1',4-dibutyl-4'-(dibromomethylene) spiro[furan-2(5H),6'-[3]oxabicyclo-[3.2.0]heptane]-2',5'-dione* (6): m.p. 105.5–106°C; IR ( $CCl_4$ ) 1830, 1800  $cm^{-1}$ ;  $^{13}C$ -NMR  $\delta_C$  165.46, 138.62, 137.59, 131.32, 81.33, 63.79, 61.70, 60.48, 37.55, 28.54, 26.28, 25.85, 22.60, 13.68. A number of resonances were not observed due to the small quantity of material available. UV  $\lambda_{max}$  acetonitrile ( $\epsilon$ ) 238nm (14900).

*(Z,Z)-5,5'-(1,2-Dibromo-1,2-ethanediylidene)bis[4-bromo-3-butyl-2(5H)-furanone]* (9): IR ( $CCl_4$ ) 1790  $cm^{-1}$ ;  $^{13}C$ -NMR ( $CDCl_3$ )  $\delta_C$  164.85, 148.06, 138.29, 127.58, 102.16, 28.93, 25.56, 22.39, 13.66; UV  $\lambda_{max}$  acetonitrile ( $\epsilon$ ) 292nm (26000).

#### Crystallography.

The data for compounds (6) and (9) were collected with a Nicolet XRD P3 single crystal four circle diffractometer, using Mo K $\alpha$  (0.71069 Å) radiation from a crystal monochromator, while those for compounds (7), (10) and (11) were collected with a Hilger and Watts four circle diffractometer using Ni-filtered Cu K $\alpha$  (1.5418 Å) radiation. Data were collected at 298 K, except for (6) and (9) which were collected at 173 K. The space group was, in each case, determined unambiguously as a result of the structure analyses reported below, but initially indicated by systematic absences of the appropriate reflections. The cell parameters were determined by a least-squares refinement of the setting angles of 12 or 25 accurately centred high angle reflections for the Hilger and Picker diffractometers respectively.

The structures were solved by Patterson and difference Fourier syntheses, except for (6) and (9) which were solved by direct methods and difference Fourier syntheses. Blocked cascade least-squares (SHELXTL) and full-matrix (SHELX76)<sup>16</sup> refinements were employed, the reflection weights being  $1/[\sigma^2(F) + g(F^2)]$ . The function minimized was  $\sum w(|F_o| - |F_c|)^2$ . Anomalous dispersion corrections were from Cromer and Liberman.<sup>17</sup> Hydrogen atoms were included as rigid groups pivoting about their carbon atoms, and all non-hydrogen atoms were assigned anisotropic thermal parameters. Numerical absorption corrections were applied in each case. The final electron density maps showed no significant residual electron density, and there were no abnormal discrepancies between observed and calculated structure factors.<sup>18</sup>

#### Crystal Data.

*3,5',7',7'',-Tetrabromo-1',4-dibutyl-4'-(dibromomethylene) spiro[furan-2(5H),6'-[3]oxabicyclo-[3.2.0]heptane]-2',5'-dione* (6) ·  $C_{18}H_{18}Br_4O_4$ , M 778, triclinic, space group  $P\bar{1}$ , a 7.510(1), b 8.134(1), c 19.608(1) Å,  $\alpha$  99.12(1),  $\beta$  98.45(1),  $\gamma$  97.38(1)°, U 1155.59 Å<sup>3</sup>,  $D_c$  2.24 g cm<sup>-3</sup>, Z 2,  $\mu$  (Mo K $\alpha$ ) 103.47 cm<sup>-1</sup>. The crystal was of approximate dimensions 0.58 x 0.20 x 0.18mm; number of independent reflections measured 2679, number with  $I > 3\sigma(I)$  2091; an unique data set was collected using 2 $\theta$ – $\omega$  scans to 2 $\theta$  83°; F(000) 736; g 0.0011; R 0.046; ratio of transmission factors 1.984.

( $\pm$ )-*trans*-4,10,11,11,12,12-hexabromo-3,9-dibutyl-1,7-dioxadispiro [4.0.4.2] dodeca-3,9-diene-2,8-dione (7) -  $C_{18}H_{18}Br_6O_4$ , M 778, monoclinic, space group C2/c,  $a$  15.983(1),  $b$  12.065(2),  $c$  13.774(1) Å,  $\beta$  115.070(1)°,  $U$  2410.74 Å<sup>3</sup>,  $D_m$  2.13 g cm<sup>-3</sup> (measured by flotation in ZnI<sub>2</sub> solution),  $D_c$  2.14 g cm<sup>-3</sup>,  $Z$  4,  $\mu$  (Cu K $\alpha$ ) 116.98 cm<sup>-1</sup>. The crystal was of approximate dimensions 0.38 x 0.10 x 0.13 mm. Number of independent reflections measured 1162, number with  $I > 3\sigma(I)$  969; a unique data set was measured to  $2\theta$  106° with  $2\theta$ - $\omega$  scans;  $F(000)$  1472,  $g$  0.0038,  $R$  0.0792; ratio of transmission factors 1.970.

(*Z,Z*)-5,5'-(1,2-Dibromo-1,2-ethanediyliene)bis[4-bromo-3-butyl-2(5H)-furanone] (9) -  $C_{18}H_{18}Br_4O_4$ , M 618, triclinic, space group  $P\bar{1}$ ,  $a$  7.728(1),  $b$  10.030(1),  $c$  14.048(2) Å,  $\alpha$  82.52(1),  $\beta$  81.45(1),  $\gamma$  86.75(1)°,  $U$  1066.82 Å<sup>3</sup>,  $D_c$  1.93 g cm<sup>-3</sup>,  $Z$  2,  $\mu$  (Mo K $\alpha$ ) 74.92 cm<sup>-1</sup>. The crystal was of approximate dimensions 0.16 x 0.52 x 0.09 mm. Number of independent reflections measured 2795, number with  $I > 3\sigma(I)$  2276; an unique data set was collected using  $\omega$  scans to  $2\theta$  90°;  $F(000)$  596;  $g$  0.0013;  $R$  0.059; ratio of transmission factors 1.827.

*cis*-4,11,12,13-Tetrabromo-3,10-dibutyl-1,6,8-trioxadispiro[4.1.4.2]trideca-3,10,12-triene-2,9-dione (10) -  $C_{18}H_{18}O_5Br_4$ , M 634, monoclinic, space group  $P2_1/c$ ,  $a$  16.682(2),  $b$  16.297(1),  $c$  8.133(1) Å,  $\beta$  90.60(1)°,  $U$  2210.97 Å<sup>3</sup>,  $D_c$  1.91 g cm<sup>-3</sup>,  $Z$  4,  $\mu$  (Cu K $\alpha$ ) 85.71 cm<sup>-1</sup>. The crystal was of approximate dimensions 0.45 x 0.75 x 0.5 mm. Number of independent reflections measured 2041, number with  $I > 3\sigma(I)$  1224; a unique data set was measured to  $2\theta$  100° with  $2\theta$ - $\omega$  scans;  $F(000)$  1224,  $g$  0.0038,  $R$  0.052; ratio of transmission factors 2.808.

( $\pm$ )-*cis*-4,10,11,11,12,12-hexabromo-3,9-dibutyl-1,7-dioxadispiro[4.0.4.2]dodeca-3,9-diene-2,8-dione (11) -  $C_{18}H_{18}O_4Br_6$ , M 778, triclinic, space group  $P\bar{1}$ ,  $a$  11.16(1),  $b$  12.65(1),  $c$  9.11(1) Å,  $\alpha$  109.6(1),  $\beta$  87.0(1),  $\gamma$  100.5(1)°,  $U$  1191.5 Å<sup>3</sup>,  $D_c$  2.17 g cm<sup>-3</sup>,  $Z$  2,  $\mu$  (Cu K $\alpha$ ) 125.78 cm<sup>-1</sup>. The crystal was of approximate dimensions 0.25 x 0.23 x 0.05 mm. Number of independent reflections measured 1307, number with  $I > 3\sigma(I)$  972; an unique data set was collected using  $2\theta$ - $\omega$  scans to  $2\theta$  80°;  $F(000)$  736,  $g$  0.004;  $R$  0.1186.

*Verification of the biological origin of the compounds in D. elegans.* *D. elegans* was collected on 19th March 1982 at St Kilda Rocks, Kaikoura, from a depth of fifteen metres, and placed in a black polythene bag before being brought to the surface. It was stored in seawater at 0°C until the following day when it was homogenised with dichloromethane. The green dichloromethane layer was separated from the red-grey aqueous emulsion by centrifugation, then filtered (Millipore 0.7µ) and analysed by HPLC (cyanopropyl column 4.5 x 250mm; hexane/propan-2-ol 5%; 220nm; flow program 0-2.5 mins at 0.7 ml/min, 2.5-5.0 mins linear increase from 0.7 to 2.5 ml/min, 5-10 mins at 2.5 ml/min). The entire process was performed in darkness to prevent the possibility of photodimerisation. No significant qualitative differences between this and other extracts were observed.

*Comparison of Collections.* Samples of *D. elegans* were collected on 3rd December 1980, 25th February 1981, 2nd April 1981, 14th January 1982, 19th March 1982 and April 1983, from St Kilda Rocks, Kaikoura, and on the 27th November 1981 from South Bay, Kaikoura.

In each case the samples were frozen for storage, and subsequently air dried, milled, and extracted with dichloromethane in a Soxhlet apparatus. The crude extracts were filtered through short columns of "Florisil" (20 x 30mm), in ten column volumes of ether. The oils obtained were dissolved in hexane/propan-2-ol (4%), filtered (Millipore 0.7µ) and analysed by HPLC (cyanopropyl column 4.5 x 250mm; hexane/propan-2-ol 5%; 220nm). No qualitative differences were observed between the chromatograms.

#### ACKNOWLEDGEMENTS

We thank Dr L.K. Pannell, NIDDK, National Institutes of Health, Bethesda for the HREIMS spectra, Kratos Ltd for the FABMS spectra, and Professor K.L. Rinehart Jr. of the University of Illinois, Urbana, for the FDMS spectra. Also the New Zealand Universities Grants Committee and the Evans Fund for financial support and Dr N.B. Perry, University of Canterbury, for proof reading.

#### References

1. McConnell, O.J., and Fenical, W., 'Antimicrobial Agents from Red Marine Algae of the Family Bonnemaisoniaceae', in 'Marine Algae: Pharmaceutical Science' (Eds Hoppe, H.A., Levring, T., and Tanaka, Y.) p 403 (de Gruyter: Berlin 1979).
2. Woolard, F.X., and Moore, R.E., *Tetrahedron* 32, 2843 (1976).
3. McConnell, O.J., and Fenical, W., *Phytochemistry* 16, 367 (1977).
4. Burreson, B.J., Moore, R.E., and Roller, P., *Tetrahedron Lett.* 473 (1975).
5. Kazlauskas, R., Lidgard, R.O., and Wells, R.J., *Tetrahedron Lett.* 3165 (1978).
6. Kazlauskas, R., Murphy, P.T., Quinn, R.J., and Wells, R.J., *Tetrahedron Lett.* 37 (1977).
7. Pettus, J.A., Wing, R.M., and Sims, J.J., *Tetrahedron Lett.* 41 (1977).
8. Rose, A.F., Pettus, J.A., and Sims, J.J., *Tetrahedron Lett.* 1847 (1977).
9. Stierle, D.B., Wing, R.M., and Sims, J.J., *Tetrahedron Lett.* 4455 (1976).
10. Alworth, W.L., 'Stereochemistry and its Application in Biochemistry', p 4 (Wiley-Interscience: New York 1972).
11. Walker, R.P., Faulkner, D.J., Van Engen, D., and Clardy, J., *J. Am. Chem. Soc.* 103, 6772 (1981).
12. Riddick, J.A., and Toops, E.E., "Organic Solvents: Physical Properties and Methods of Purification", 2nd Edn (Wiley Interscience: New York 1955).
13. Patt, S.L. and Shooley, J.N., *J. Magn. Reson.* 46, 535 (1982).
14. Norton, R.S., *Tetrahedron* 33, 2577 (1977).
15. Bremser, W., Ernst, L., Franke, B., Gerhards, R., Hardt, A., "Carbon-13 NMR Spectral Data" (Verlag Chemie: Weinheim 1981).
16. Sheldrick, G.M., "SHELXTL User Manual", Revision 4.1 (1981) Nicolet XRD Corporation, Cupertino, California, U.S.A., and Sheldrick, G.M., "Program for Crystal Structure Determination" (University of Cambridge: England 1976).
17. Cromer, D.T., and Liberman, D., *J. Chem. Phys.* 53, 1891 (1970).
18. Atomic coordinates for this structure have been deposited with the Cambridge Crystallographic Data Centre. The coordinates can be obtained on request from The Director, Cambridge Crystallographic Data Centre, University Chemical Laboratory, Lensfield Road, Cambridge CB 1EW, U.K. Supplementary data available: tables for the observed and calculated structure factors and tables of temperature factors. See Notice to Authors *Tetrahedron* 40(2), ii (1984).

*Proofs to: Dr J W Blunt, Department of Chemistry, University of Canterbury,  
Christchurch, New Zealand.*

*Short Title: NMR Spectra of Thyransferyl Acetate*

# **Complete Assignment of the $^{13}\text{C}$ and $^1\text{H}$ NMR Spectra of Thyransferyl Acetate**

**John W Blunt,\* John D McCombs, Murray H G Munro  
and Frank N Thomas**

Department of Chemistry, University of Canterbury, Christchurch,  
New Zealand.

## **ABSTRACT**

The assignment of all resonances in the  $^{13}\text{C}$  and  $^1\text{H}$  nmr spectra of thyransferyl acetate has been made through the application of 2D nmr experiments, together with the use of difference nOe spectroscopy.

**KEY WORDS:** Thyransferol Thyransferyl acetate NOE Spectral assignments

## **INTRODUCTION**

Since its discovery in 1975, the marine algal squalene-derived polyether metabolite thyransferol (1a)<sup>1</sup> has generated a high degree of interest, with reports of derivatives having potent cytotoxic effects,<sup>2,3</sup> the characterisation of venustatriol (isomeric with thyransferol at C18 and C19),<sup>4</sup> and the various synthetic efforts directed towards these compounds.<sup>5-9</sup> Although some nmr data for these compounds have been reported, no complete assignments of the  $^{13}\text{C}$  and  $^1\text{H}$  nmr spectra have been made. We now report the complete assignment of the spectra of thyransferyl acetate (1b), and the partial assignment of the spectra of thyransferol (1a), through the application of a range of 2D nmr methods, together with difference nOe spectroscopy.

## **ASSIGNMENTS**

Assignment of all hydrogen and carbon resonances in the nmr spectra of thyransferyl acetate (1b) were made following the acquisition of homonuclear (COSY), heteronuclear (HETCOR) and long-range heteronuclear (XCORFE)<sup>10</sup> 2D chemical shift correlation nmr spectra, and nOe difference nmr spectra. The starting points for the assignments were the unique resonances at 58.95 ppm in the  $^{13}\text{C}$  nmr spectrum for the brominated carbon C3, and at 4.90 and 2.08 ppm in the  $^1\text{H}$  nmr spectrum for H18 and  $\text{CH}_3\text{CO}$ , respectively. From these resonances, pathways of connectivities could be traced using the 2D nmr and nOe difference spectra. The connectivities observed, and which permitted the assignments to be made, are shown in

Figure 1. The assignments are shown for the 'Assignment To' row values based on the connectivity as indicated from a previously assigned value in the 'Assignment From' column. For example, starting with C3 (uniquely defined by its chemical shift at 58.95 ppm) in the 'From' column, this is seen to be related by a HETCOR correlation to a proton at 3.89 ppm in the 'To' row which must therefore be H3. Returning to the 'From' column, the now assigned H3 at 3.89 ppm is seen to be correlated via COSY connections with protons at 2.10 and 2.25 ppm in the 'To' row, thus making the assignment of the H2 protons. Starting with these in the 'From' column, a HETCOR connection to a carbon signal at 28.19 ppm in the 'To' row gives the assignment for C4. Other assignments (Table 1) were established in a similar manner, starting with the unique assignment for C3 as described above, and from H18 at 4.90 ppm.

No distinction has been made between 2- and 3-bond long-range heteronuclear couplings from the XCORFE experiment, but in all cases the connectivities shown are unambiguous. Considerable importance has been attached to the results from difference nOe spectra. By this technique every *pro-r* and *pro-s* methylene hydrogen resonance was unambiguously assigned. Molecular mechanics calculations<sup>11</sup> revealed a number of accessible conformations for the C14-C19 region, the majority of which had conformations for the C16-C17-C18 bonds as found in the crystal structure<sup>1</sup> for 1a (Figure 2). This finding permitted the assignment for H16s by noting a strong nOe interaction between one of the H16 resonances at 1.25 ppm and the H18 resonance at 4.90 ppm. The boat conformation for ring C as found in

the solid state (Figure 2) is also confirmed in the solution state structure with the observation of an nOe interaction between H11 and H14 (see inset for Figure 3). As shown in Figure 2, H7, H11 and H14 form a linear three-spin system, as irradiation of the H7 resonance produces a negative nOe effect for H14, and *vice versa*, while irradiation of each of the H7 and H14 resonances creates a positive nOe effect for H11. These effects had previously been observed for a synthetic fragment of thyrsiferol.<sup>6</sup> While the <sup>1</sup>H nmr chemical shift values for methylene hydrogen resonances in the 1.0-2.2 ppm region have been determined from the HETCOR experiment, it has not been possible to extract individual coupling constants for each methylene hydrogen due to the complexity of this region. An attempt to achieve some resolution of the overlapping multiplets by means of a 300 MHz homonuclear J-resolved spectrum was not successful, presumably because of second order effects.

With the completion of the assignment of the spectra for thyrsiferol 18-acetate (1b), the assignments of the spectra of thyrsiferol (1a) were straightforward (Table 1), with differences being noted only for those positions adjacent to C18. While some of these differences would be expected following the change from an acetoxyl to an hydroxyl substituent at C18, changes at somewhat more remote positions (*eg* C13 and C20) suggest that the conformations of the bridging section C14 to C19 are different between 1a and 1b. It has not been possible to fully assign the methylene proton resonances of thyrsiferol (1a) as the HETCOR spectrum for the small amount of this sample available did not reveal all of the carbon-hydrogen correlations.

## EXPERIMENTAL

A sample of thyrsiferol (1a) was obtained from *Laurencia thyrsifera*, and its 18-acetyl derivative (1b) prepared by reaction with acetic anhydride in pyridine.<sup>1</sup> The spectra for thyrsiferol (1a) (8 mg) and its acetate (1b) (20 mg) were obtained for 0.6 ml CDCl<sub>3</sub> solutions in a Varian XL300 spectrometer fitted with a 5mm broad-band switchable probe operating at 23°C. <sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded at 300 MHz and 75 MHz respectively. DEPT spectra were obtained with the standard Varian DEPT sequence. COSY spectra were obtained in both the absolute value mode and in the double quantum filtered phase sensitive mode using standard Varian pulse sequences with spectral windows of 1300 Hz, 512 increments for F<sub>1</sub>, F<sub>2</sub> acquisition times of 0.395 s, and presequence delays of 1 s. The HETCOR experiments were obtained in the absolute value mode using the standard Varian pulse sequence, with <sup>13</sup>C SW=5600 Hz, <sup>1</sup>H SW=1300 Hz, 128 increments of F<sub>1</sub>, an F<sub>2</sub> acquisition time of 0.2 s, and a presequence delay of 1 s. The XCORFE experiment for 1b was performed as described by Reynolds *et al*,<sup>10</sup> with a T value of 0.128 s followed by a second experiment with T=0.07 s, and other parameters as given for the HETCOR experiment. The connectivities established by this method were subsequently confirmed by a proton-detected HMBC experiment,<sup>12</sup> with the delay optimised for 8.3 Hz couplings. NOe difference spectra were obtained using the low-power cycling method of Kinns and Sanders,<sup>13</sup> modified as described previously,<sup>14</sup> and employing irradiation times of 2 s followed by data acquisition for 1 s.

## ACKNOWLEDGEMENT

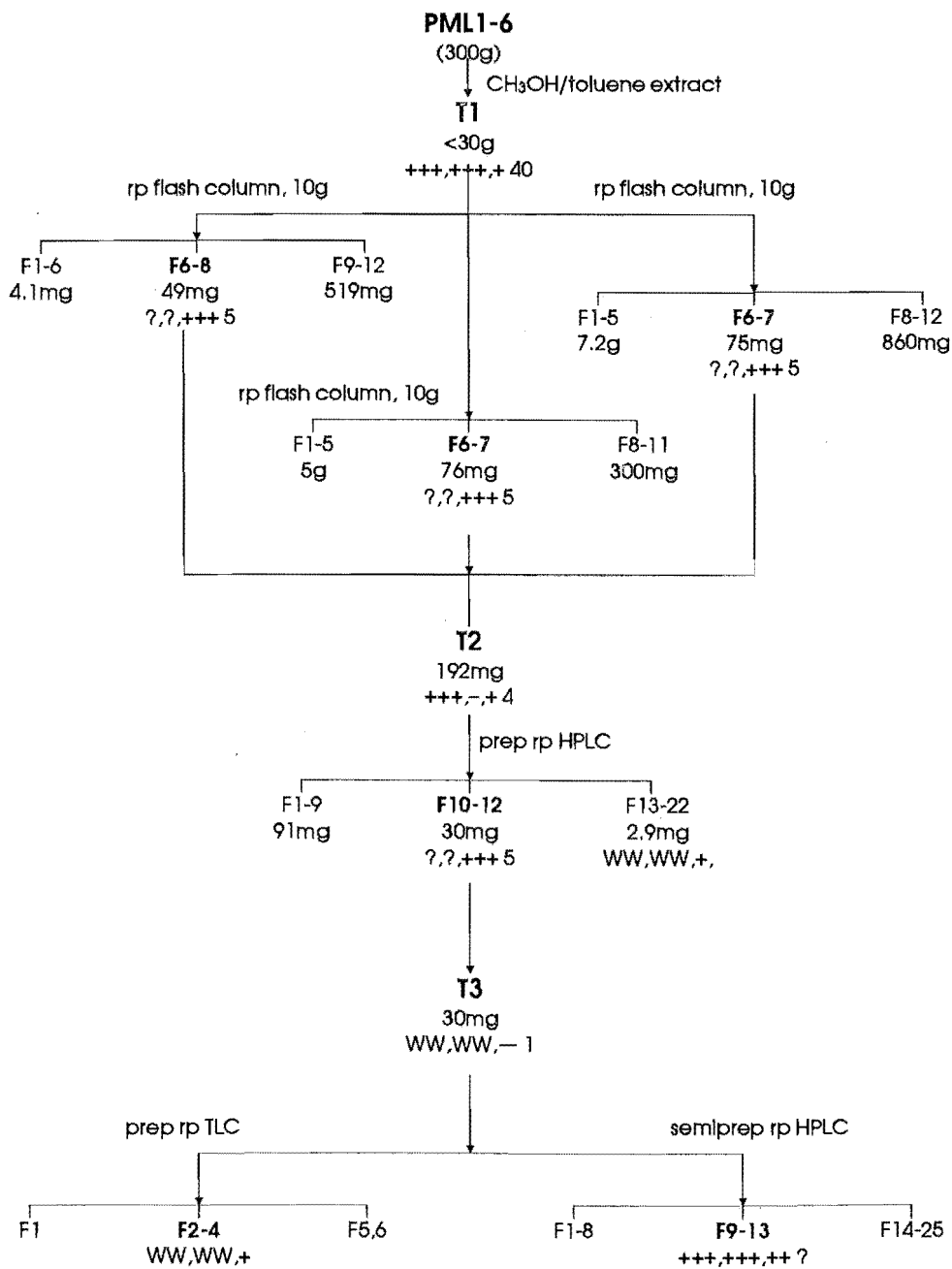
We thank the New Zealand Universities Grants Committee for support for the purchase of equipment used in this work.

## REFERENCES

1. J. W. Blunt, M. P. Hartshorn, T. J. McLennan, M. H. G. Munro, W. T. Robinson and S. C. Yorke, *Tetrahedron Lett.* 69 (1978).
2. T. Suzuki, M. Suzuki, A. Furusaki, T. Matsumoto, A. Kato, Y. Imanaka and E. Kurosawa, *Tetrahedron Lett.* 26, 1329 (1985).
3. T. Suzuki, S. Takeda, M. Suzuki, E. Kurosawa, A. Kato and Y. Imanaka, *Chemistry Lett.* 361 (1987).
4. S. Sakemi, T. Higa, C. W. Jefford and G. Bernardinelli, *Tetrahedron Lett.* 27, 4287 (1986).
5. C. A. Broka, L. Hu, W. J. Lee and T. Shen, *Tetrahedron Lett.* 28, 4993 (1987).
6. M. Hashimoto, T. Kan, M. Yanagiya, H. Shirahama and T. Matsumoto, *Tetrahedron Lett.* 28, 5665 (1987).
7. M. Hashimoto, T. Kan, K. Nozaki, M. Yanagiya, H. Shirahama and T. Matsumoto, *Tetrahedron Lett.* 29, 1143 (1988).
8. E. J. Corey and D-C. Ha, *Tetrahedron Lett.* 29, 3171 (1988).
9. C. A. Broka and Yi-Tsong Lin, *J. Org. Chem.* 53, 5876 (1988).

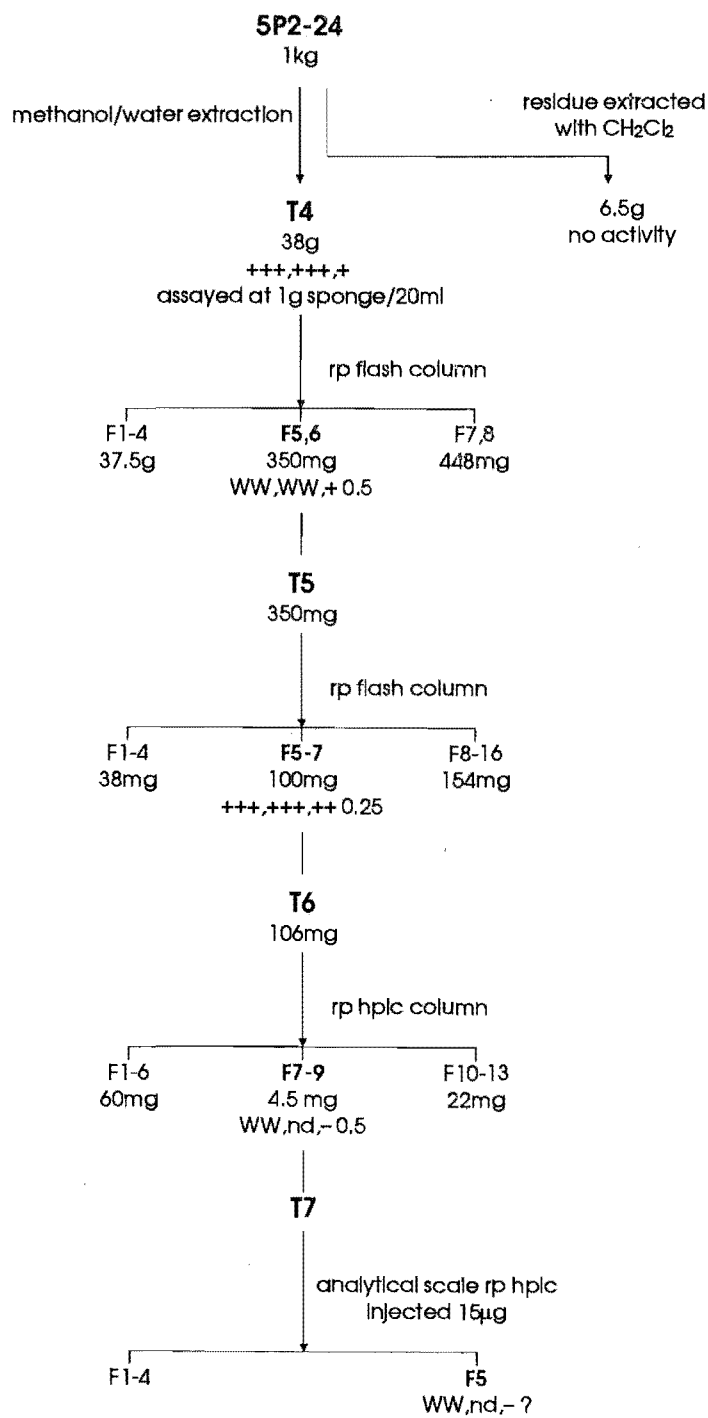
10. W. F. Reynolds, D. W. Hughes, M. Perpich-Dumont and R. G. Enriquez, *J. Magn. Reson.* **64**, 303 (1985).
11. Calculations performed using the MM2 MODEL program of C. Still, extensively rewritten by K. Steliou.
12. A. Bax and M. F. Summers, *J. Am. Chem. Soc.* **108**, 2093 (1986).
13. M. Kinns and J. K. M. Sanders, *J. Magn. Reson.* **56**, 518 (1984).
14. N. B. Perry, J. W. Blunt and M. H. G. Munro, *Magn. Reson. Chem.* in press (1989).

## Separation Tree for the PML1-6 Extract

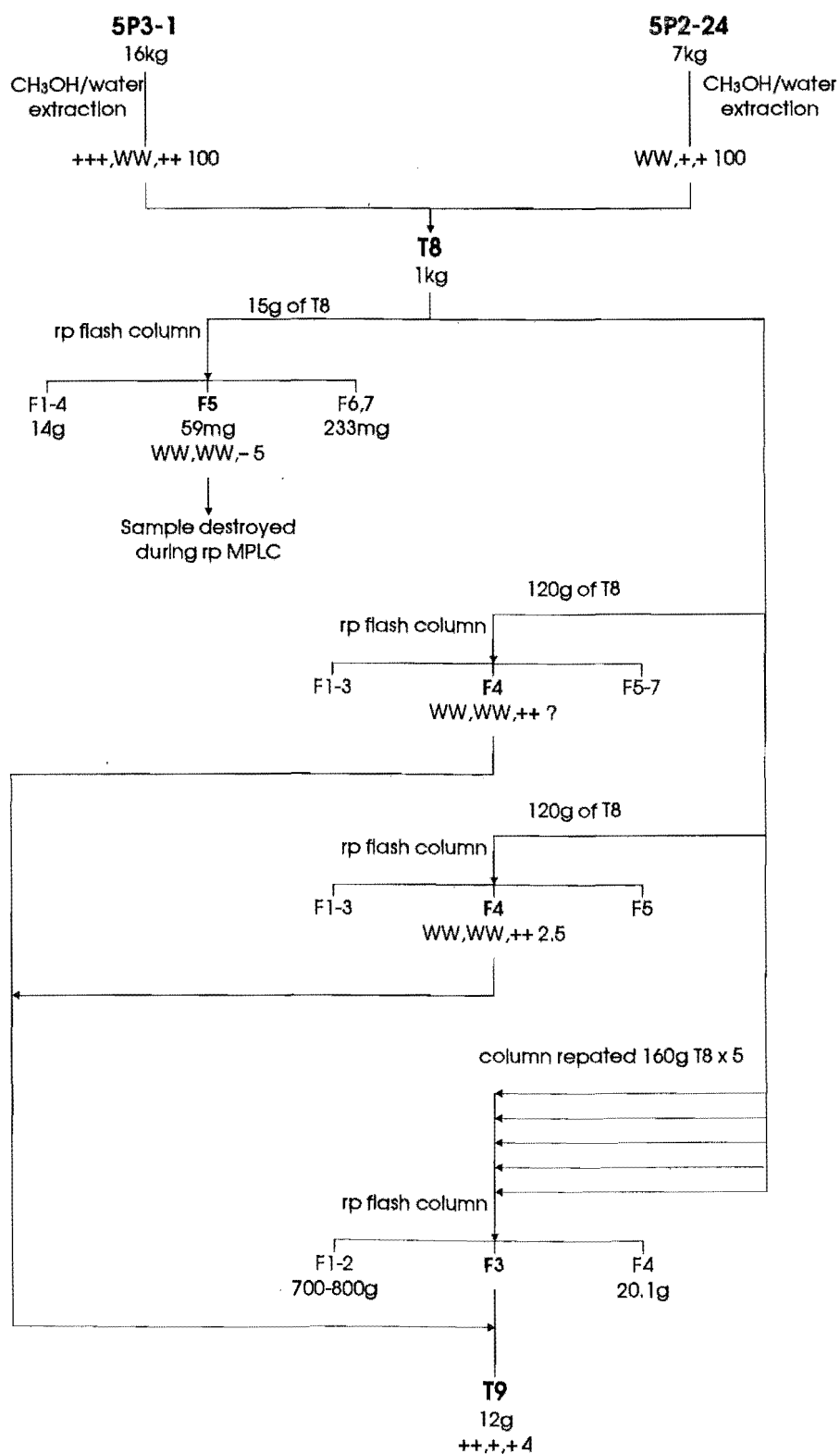


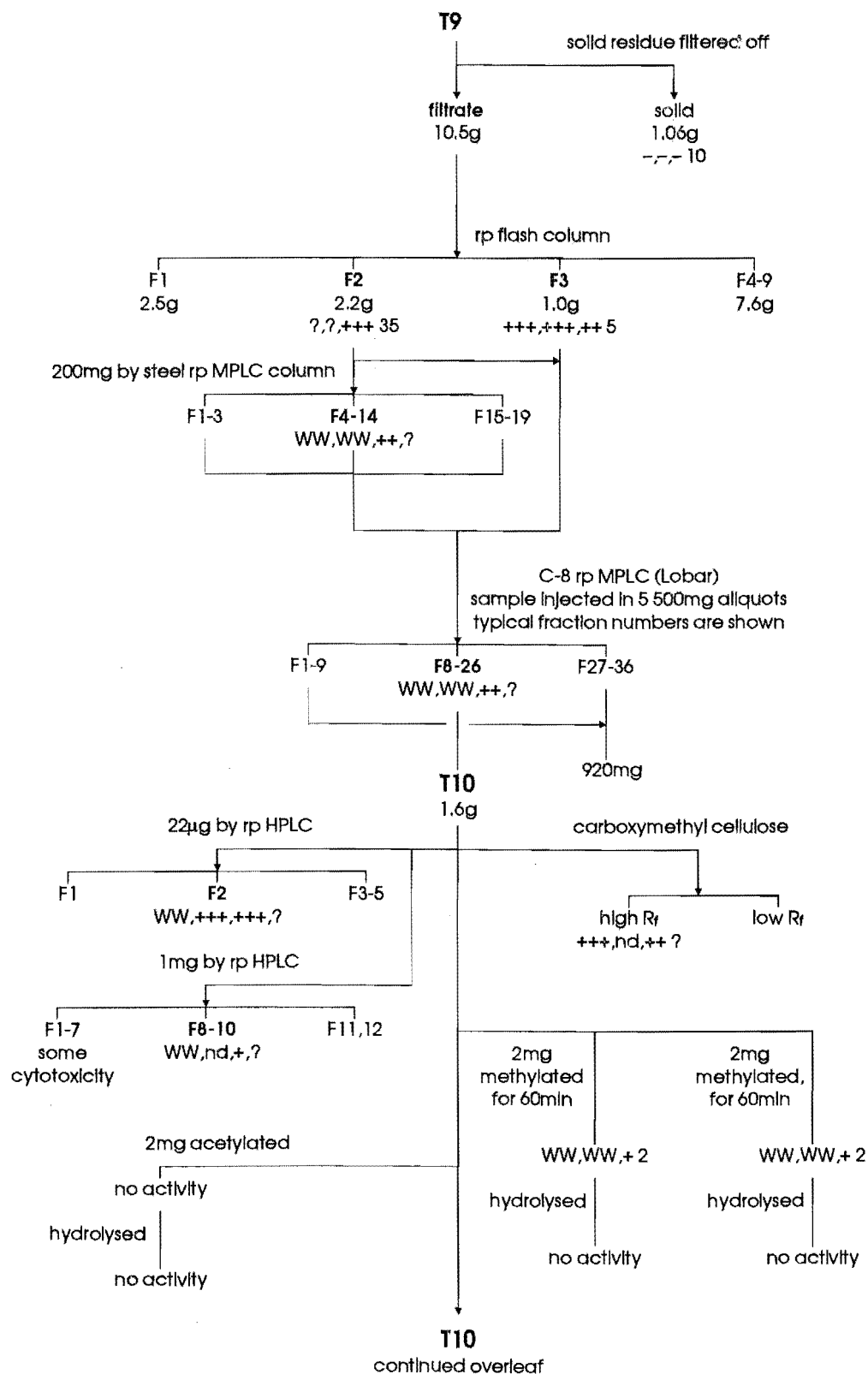


## Separation Tree for 1kg Extract of 5P2-24

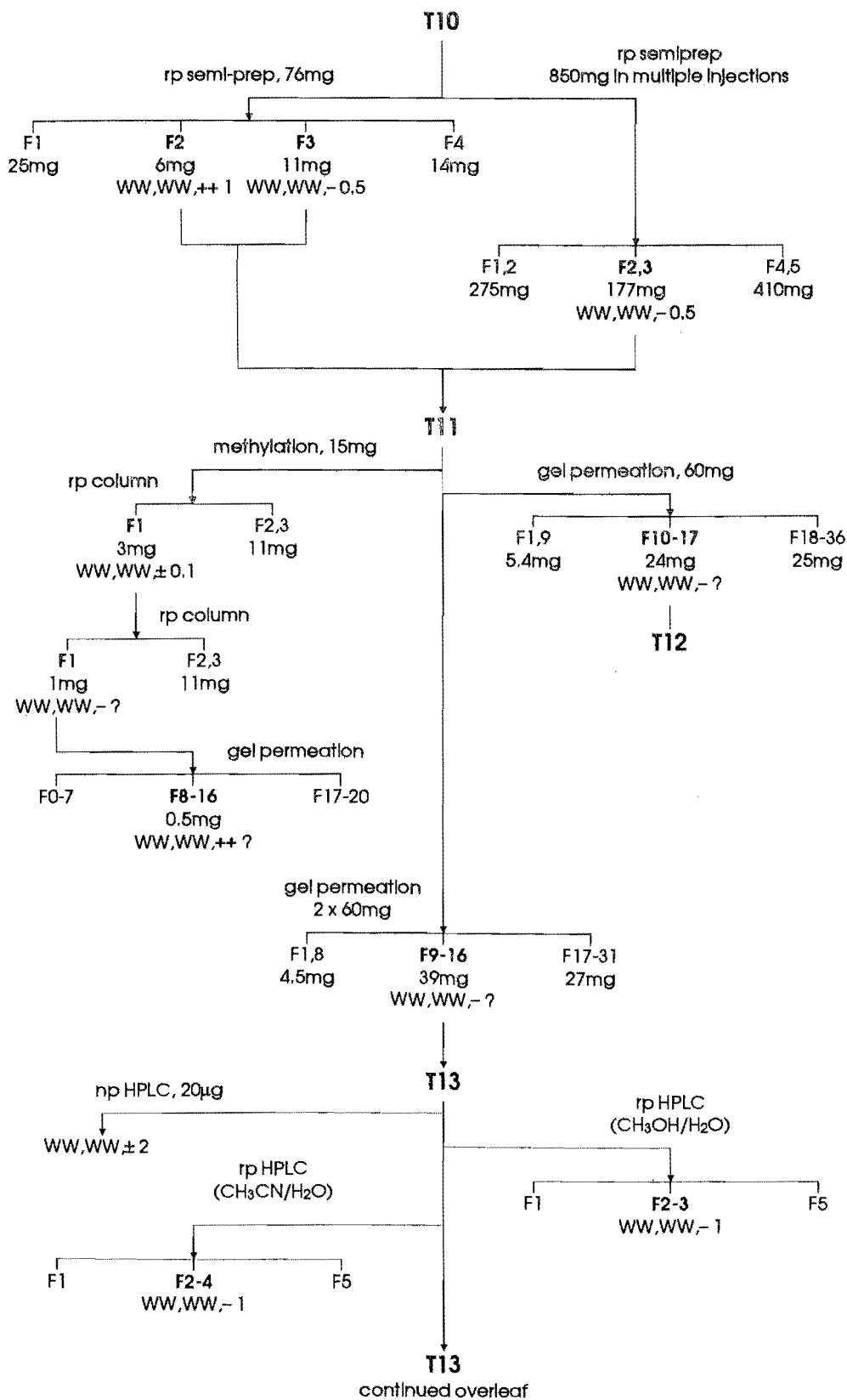


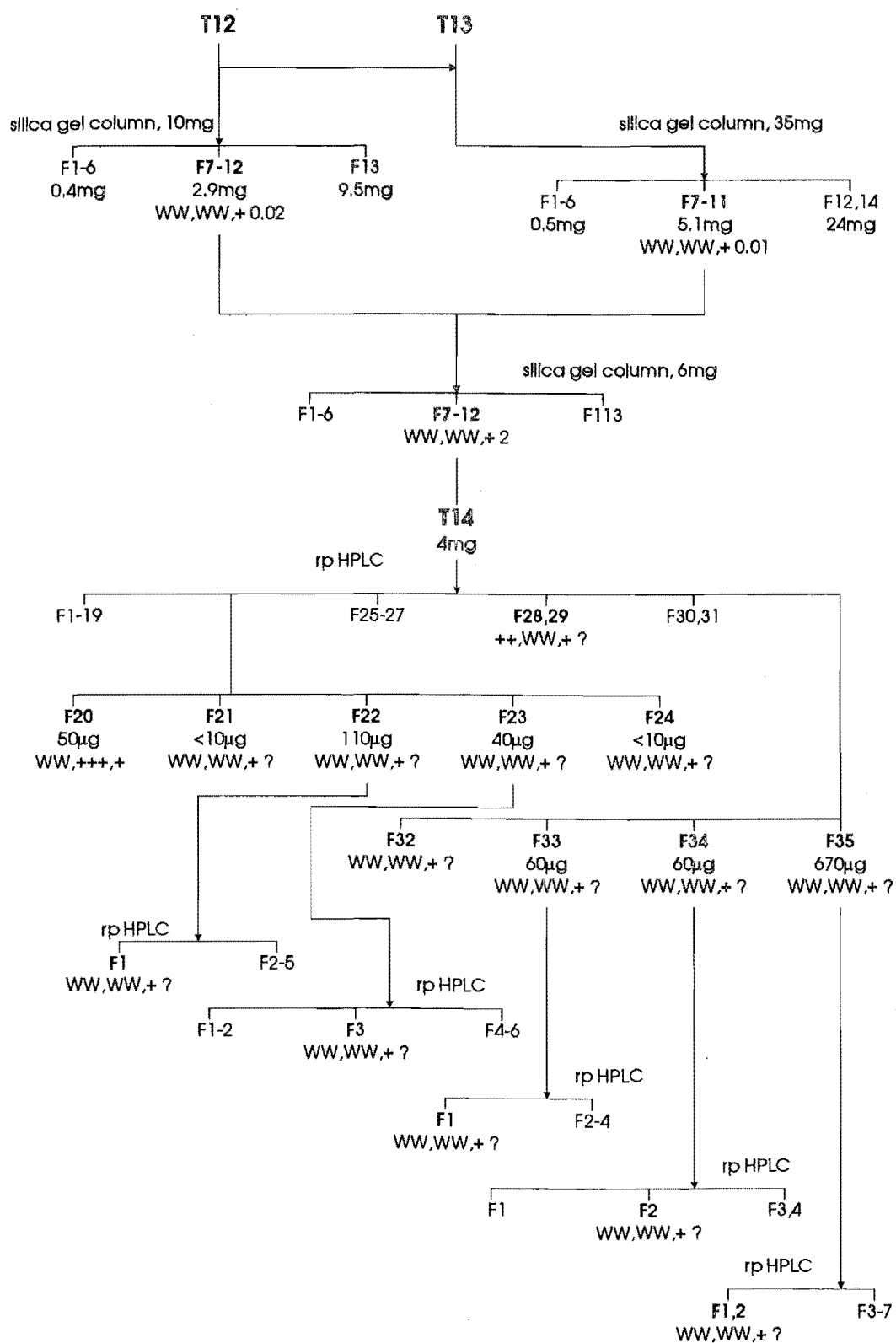
## Separation Trees for the Extract of 5P2-24 and 5P3-1





Discussion - Tedania





# NMR Correlation Table for Thyriferol 18-Acetate

		Assignment To																															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	
Assignment From	$\delta$ C $\delta$ H																																
1	23.66	H																															
2	74.94		X																														
3	58.95			H																													
4	28.19																																
5	37.07																																
6	74.34																																
7	86.50																																
8	22.94																																
9	38.53																																
10	71.88																																
11	76.24																																
12	21.07																																
13	23.80																																
14	75.82																																
15	72.95																																
16	32.08																																
17	20.59																																
18	78.41																																
19	84.11																																
20	34.77																																
21	26.11																																
22	86.98																																
23	70.39																																
24	24.06																																
25	30.98																																
26	20.02																																
27	21.37																																
28	23.09																																
29	23.03																																
30	27.48																																
31	21.11																																
	2.08																																

The path by which the assignments were made is shown by tracing from previously assigned atoms, in the "Assignments From" column, to atoms in the "Assignments To" row. The type of correlation is indicated by the following symbols: C = COSY, ▲ = nOe, H = HETCOR, X = XCORFE, ■ = COSY + nOe

### Crystal Structure of Thysiferyl 18-Acetate

